

Original Article

Evaluation of HLA typing by next generation sequencing from proficiency testing data at Siriraj Hospital

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Abstract:

Introduction: HLA gene typing has been greatly improved by DNA sequencing using next generation sequencing (NGS), a high-resolution assay that is now being used in many laboratories. HLA gene testing plays an important role in hematopoietic stem cell transplantation and organ transplantation by selecting suitable donors for recipients. **Objective:** To evaluate the results of HLA typing by NGS method using data obtained from proficiency testing programs. **Materials and Methods:** The One Lambda, Inc., USA, AllType™ NGS assay kit and the Ion S5™ XL system instrument were used to examine 48 DNA samples from the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University in proficiency testing programs. **Results:** HLA-A, B, C, DRB1, DRB3/4/5, DQA1, DQB1, DPA1 and DPB1 typing results were concordant with the results of proficiency testing in HLA-A, B, C, DRB3/4/5, DQB1, DPA1, and DPB1 at 100% of all three institutions which the results were compared at high resolution at the HLA second field level. Only one sample was shown in the HLA first field level of HLA-DRB1, DQB1, and DPB1 typing. **Conclusion:** The HLA gene NGS assay using the AllType™ NGS assay and the Ion S5™ XL system yielded results concordant with proficiency testing results that can detect both HLA class I and class II genes.

Keywords : ● HLA ● Genes ● Next generation sequencing ● Proficiency testing

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นิพนธ์ต้นฉบับ

การประเมินผลการตรวจเอชแอลเอโดยวิธี next generation sequencing จากข้อมูลของการทดสอบความชำนาญที่โรงพยาบาลศิริราช

โกมล หลวงตระกูล และ ศศิจิต เวชแพศย์

ภาควิชาเวชศาสตร์การธนาคารเลือด คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

บทคัดย่อ

คำนำ การตรวจ HLA ยีนมีการพัฒนาเทคโนโลยีอย่างมากในการหาลำดับเบสของดีเอ็นเอโดยใช้วิธี next generation sequencing (NGS) ซึ่งเป็นการตรวจที่มีความละเอียดสูงโดยกำลังจะถูกนำมาใช้ในหลายห้องปฏิบัติการที่มีการตรวจทางด้าน HLA ยีนโดยที่ HLA มีบทบาทสำคัญสำหรับการปลูกถ่ายเซลล์ต้นกำเนิดเม็ดโลหิตและปลูกถ่ายอวัยวะในการคัดเลือกผู้บริจาคให้เหมาะสมกับผู้รับ **วัตถุประสงค์** เพื่อประเมินผลการตรวจ HLA typing ด้วยวิธี NGS โดยใช้ข้อมูลที่ได้จากการทดสอบความชำนาญ (proficiency testing) **วัสดุและวิธีการ** ใช้ชุดตรวจของ AllType™ NGS assay ของบริษัท One Lambda, Inc., USA โดยใช้เครื่อง Ion S5™XL system มาตรวจกับตัวอย่างดีเอ็นเอจากภาควิชาเวชศาสตร์การธนาคารเลือด คณะแพทยศาสตร์ศิริราชพยาบาลมหาวิทยาลัยมหิดล ที่เข้าร่วมการทดสอบความชำนาญ (proficiency testing) จำนวน 48 ราย **ผลการศึกษา** พบว่าผลตรวจ HLA-A, B, C, DRB1, DRB3/4/5, DQA1, DQB1, DPA1 และ DPB1 มีความสอดคล้องกับผลของ proficiency testing ใน HLA-A, B, C, DRB3/4/5, DQB1, DPA1 และ DPB1 ที่ 100% ของทั้งสามสถาบันโดยที่ผลการตรวจมีการเปรียบเทียบผลในระดับ high resolution ที่ HLA field ที่ 2 มีเพียง 1 ตัวอย่างที่ HLA-DRB1, DQB1, DPB1 ที่ผลออกมาใน HLA field ที่ 1 **สรุป** การตรวจ HLA ยีนโดยวิธี NGS ด้วยชุดตรวจของ AllType™ NGS assay โดยใช้เครื่อง Ion S5™XL system ให้ผลที่สอดคล้องกับผลการทดสอบความชำนาญ โดยสามารถจะตรวจได้ทั้งในยีน HLA class I และ class II พร้อมกัน

คำสำคัญ : ● เอชแอลเอ ● ยีน ● การวิเคราะห์ลำดับเบสยุคใหม่ ● การทดสอบความชำนาญ

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต. 2566;33:189-98.

Introduction

Human leukocyte antigen (HLA) genes are highly polymorphic genes reported in the Immuno Polymorphism Database - International ImMunoGeneTics (IPD-IMGT)/HLA database. To date, as of April 2023, 37,247 HLA alleles had been identified¹, including HLA-A, B, and C in the HLA class I genes and HLA-DRB1, 3, 4, 5, DQA1, DOB1, DPA1, and DPB1 in the HLA class II genes. HLA typing has been greatly improved by the technology of DNA sequencing using Next Generation Sequencing (NGS), a high-resolution assay² that is being used in many HLA laboratories.^{3,4} In several previous studies HLA played an important role in hematopoietic stem cell transplantation and organ transplantation in selecting the appropriate donor for the recipient of hematopoietic stem cells and improving the graft outcomes.^{5,6} The current technology of NGS has been developed to rapidly detect the entire gene coverage of HLA loci ranging from the 5' UTR to 3' UTR regions making it less ambiguous and easier to identify new alleles. Because NGS has many platforms to choose from, many companies have different technologies, so each of which is suitable for use in different tests. Each platform is different in terms of the number of samples to be examined each time, the preparation of DNA libraries, DNA amplification for sequencing analysis, methods for sequencing analysis, the difference in the obtained base sequence length data and data accuracy levels.⁷⁻⁹ The objective of this study was to evaluate the HLA typing results of the AllType™ NGS assay of One Lambda (USA) using the Ion S5™XL system in which the samples were from the proficiency programs that the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University participated in.

Materials and Methods

Data and DNA samples were from proficiency testing programs that the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital Mahidol University

participated in. There were 48 samples divided into 12 samples from proficiency testing in Thailand, namely the proficiency testing for HLA testing for organ allocation organized by the Organ Donation Center, Thai Red Cross Society (ODC-TRCS); 36 samples from overseas institutions divided into 12 samples from the Asia-Pacific Histocompatibility and Immunogenetics Association (APHIA) Quality Assurance Program (Australia), and 24 samples from the UCLA International Cell Exchange (USA). All DNA samples were obtained from September 2016 to August 2018 from the Department of Transfusion Medicine. This research was approved by the Human Research Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University (Si 670/2021).

HLA genotyping by NGS

The number of DNA samples for each test was 24 at the same time, divided into two batches. As a first step, DNA libraries were prepared using AllType reagent kits (One Lambda, Canoga Park, California, USA) running on the Ion S5 XL platform using Thermo Fisher's reagents: the Ion 530™ Chip kit (A27763) and the Ion S5™ Ext kit. Chef Supplies (A30673) This reagent is a single multiplex polymerase chain reaction (PCR) to amplify the total number of genes in HLA-A, B, C, DQA1, DPA1 and in parts of HLA-DRB1, 3, 4, 5, DOB1, and DPB1 genes were amplified from exon 2 to 3'UTR of genes, after which they were used in library preparations, subjected to barcode ligation and size selection, then template preparation and sequencing. The data were analyzed using HLA TypeStream visual software (TSV) version 1.1.0. The percentages of accuracy for each HLA gene, including HLA-A, B, C, DRB1, DRB3/4/5, DQA1, DOB1, DPA1 and DPB1, were compared with the HLA typing summary results of the proficiency testing of each participating institution, which was the gold standard compared to the NGS method and the percentage concordance (% concordance) of each HLA allele was analyzed.

Results

Altogether, 194 alleles in the high-resolution (second field) were observed in 48 samples of proficiency testing at the three institutions using One Lambda's AllType™ NGS reagent kit. There were 98 alleles in HLA class I; 43 alleles were found in the HLA-B locus which was the most polymorphic, and the frequency of the HLA-B*07:02 allele was the highest (n = 9). For the HLA-A locus, 31 alleles were found with the highest frequency of the HLA-A*02:01 allele (n = 20), and 24 alleles were found in the HLA-C locus, with the frequency of the HLA-C*07:02 allele being the highest (n = 15). For HLA class II, 96 alleles were found, with the HLA-DRB1 locus being the most polymorphic with 34 alleles of which the HLA-DRB1*07:01 allele was the most common (n = 11),

followed by the HLA-DPB1 locus (18 alleles), of which the HLA-DPB1*04:01 allele was the most prevalent (n = 21) and the HLA-DQB1 locus (15 alleles), of which the HLA-DQB1*03:01 allele was the most prevalent (n = 21), as shown in Tables 1 and 2.

Since the proficiency testing data of twelve samples from the ODC-TRCS did not show results for HLA-DQA1, DPA1, and DPB1 alleles, these were tested by NGS in this study. The results were not compared with those of the ODC-TRCS (Table 3), but the results obtained were compared to previous studies.¹⁰ The alleles found were highly prevalent in the Thai population, except for HLA-DQA1*05:09, which can be found in the South African Worcester population.¹¹

Table 1 HLA class I alleles tested by Siriraj Hospital using the NGS method from proficiency testing samples

HLA class I alleles						HLA class I alleles					
A*	N = 96	B*	N = 96	C*	N = 96	A*	N = 96	B*	N = 96	C*	N = 96
01:01	11	07:02	9	01:02	7	30:09	1	35:41	1	17:01	1
02:01	20	07:05	2	02:02	4	31:01	3	38:01	3	18:02	1
02:03	2	08:01	6	02:10	1	32:01	4	38:02	2		
02:05	1	13:02	1	03:02	3	33:03	4	39:25N	1		
02:07	2	14:01	1	03:03	6	34:01	2	40:01	5		
02:11	1	14:02	1	03:04	7	68:01	1	40:04	2		
02:13	1	15:01	4	04:01	5	68:02	1	40:06	1		
02:30	1	15:02	1	04:03	3	74:01	1	41:01	1		
02:291	1	15:03	1	05:01	4	80:01	1	44:02	4		
03:01	9	15:13	2	06:02	6			44:03	2		
11:01	6	15:17	2	07:01	11			44:12	1		
24:02	8	15:21	1	07:02	15			46:01	2		
24:07	1	15:25	2	07:04	1			48:01	1		
24:17	1	15:35	2	07:06	1			50:01	1		
24:23	1	18:01	5	08:01	4			51:01	5		
24:37	2	27:02	1	08:02	2			54:01	2		
24:53	1	27:05	4	12:02	1			55:01	2		
25:01	1	27:05	1	12:03	6			57:01	4		
25:04	1	27:06	1	14:02	2			58:01	1		
26:01	2	35:01	2	15:02	1			81:01	1		
29:01	3	35:03	2	15:05	3			82:01	2		
29:02	2	35:04	1	16:01	1						

Table 2 HLA class II alleles tested by Siriraj Hospital using the NGS method from proficiency testing samples

HLA class II alleles											
DRB1*	N = 96	DRB3*	N = 34	DQA1*	N = 72	DQB1*	N = 96	DPA1*	N = 72	DPB1*	N = 72
01:01	7	01:01	8	01(new)	1	02:01	5	01:03	48	01:01	5
01:03	3	02:02	18	01:01	8	02:02	5	02:01	15	02:01	13
03:01	6	03:01	8	01:02	17	03:01	21	02:02	6	03:01	9
04:01	5			01:03	3	03:02	12	02:07	1	04:01	21
04:03	4	DRB4*	N = 30	01:04	1	03:03	8	03:01	2	04:02	4
04:04	3	01:01	2	01:05	2	04:01	1			05:01	4
04:05	2	01:03	23	02:01	8	04:02	2			06:01	1
04:07	1	01:03N	5	03:01	9	05:01	16			10:01	1
04:26	1			03:03	5	05:02	9			11:01	3
04:31	1	DRB5*	N = 15	04:01	2	05:03	2				
07:01	11	01:01	11	05:01	3	06:02	8			13:01	3
08:01	1	01:02	1	05:03	2	06:03	3			14:01	2
08:02	1	02:02	3	05:05	7	06:04	1			16:01	1
08:06	1			05:09	1	06:04	2			17:01	1
09:01	2			06:01	3	06:09	1			18:01	1
10:01	3									20:01	1
11:01	7									40:01	1
11:19	1									104:01	1
11:29	1										
12:01	1										
12:02	5										
13:01	4										
13:02	4										
13:10	1										
14:02	2										
14:04	1										
14:05	1										
14:54	1										
15:01	5										
15:02	5										
15:03	1										
16:01	1										
16:02	2										
16:04	1										

Table 3 HLA-DQA1/DPA1/DPB1 alleles of the ODC-TRCS proficiency testing samples using the NGS method

HLA class II alleles					
DQA1*	N=24	DPA1*	N=24	DPB1*	N=24
01:01	3	01:03	8	01:01	1
01:02	3	02:01	9	02:01	2
01:04	2	02:02	6	02:02	1
01:05	2	04:01	1	04:01	2
02:01	3			04:02	3
03:01	2			05:01	3
03:02	2			09:01	1
03:03	1			13:01	6
05:01	2			14:01	2
05:05	3			26:01	1
05:09	1			104:01	2

The NGS proficiency testing results were consistent with results of UCLA and ODC-TRCS samples in all HLA loci (100% concordance) and 100% concordance with APHIA samples in HLA-A, B, C, DRB3/4/5, DQB1, DPB1, except for HLA-DRB1, DQA1, DPA1 with 91.7% concordance (Table 4), where NGS results were detected as DRB1*04, 15, in which APHIA results were DRB1* 04:03, 15:01. For DQA1, NGS results cannot be interpreted, but APHIA results were DQA1*01:02, 03:01 and for DPA1 NGS results were interpreted as DPA1*01, 01 in which APHIA results were DPA1*01:03, 01:03.

NGS takes the form of single-strand sequencing with short strings (reads) before converging into long strings, where the number of reads produced varies. Sequences with more reads have higher sequence accuracy.⁹ Of all the samples examined by NGS, the mean number

of reads (average total reads) for each locus exceeded the acceptable minimum (> 500 reads).¹² It was found that the average number of reads for DQB1 was the highest (32,780 reads) and the lowest was for DQA1 (12,082 reads), as shown in Figure 1.

From this study, all warnings were analyzed by TypeStream visual software prior to conclusions for each locus, whereas all alerts contain the balance of allele, high background, homozygous, and mismatched introns of which HLA-DPB1 was the most notified (68/72, 94.44%), whereas HLA-A was the least notified (1/96, 1.04%) (Figure 2).

Discussion

Nowadays, HLA gene typing is performed using the NGS technique, of which there are several companies that produce ready-made assays, making it convenient for typing without the need to provide suitable primers for preparing HLA gene libraries. There are also ready-made programs for data analysis that can generate the results of HLA typing. Each company may have the same or different principles and equipment used in the examination.^{13,14} In this study, One Lambda (USA) AllType™ NGS assay kit was evaluated, and the Ion Torrent system¹⁵ was used with the Ion S5™XL system at the Division of Molecular Genetics, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

Of the 48 samples tested by NGS, the results were consistent with the results of proficiency testing except in one sample where the results of some locus tests were incomplete. However, when looking at the number of

Table 4 The percentage concordance between NGS and known HLA genotypes at the second field level of proficiency testing samples

% Concordance	A	B	C	DRB1	DRB3/4/5	DQA1	DQB1	DPA1	DPB1
APHIA samples	100	100	100	91.7	100	91.7	100	100	91.7
UCLA samples	100	100	100	100	100	100	100	100	100
Thai Red Cross Society samples	100	100	100	100	100	NA	100	NA	NA

NA= not applicable

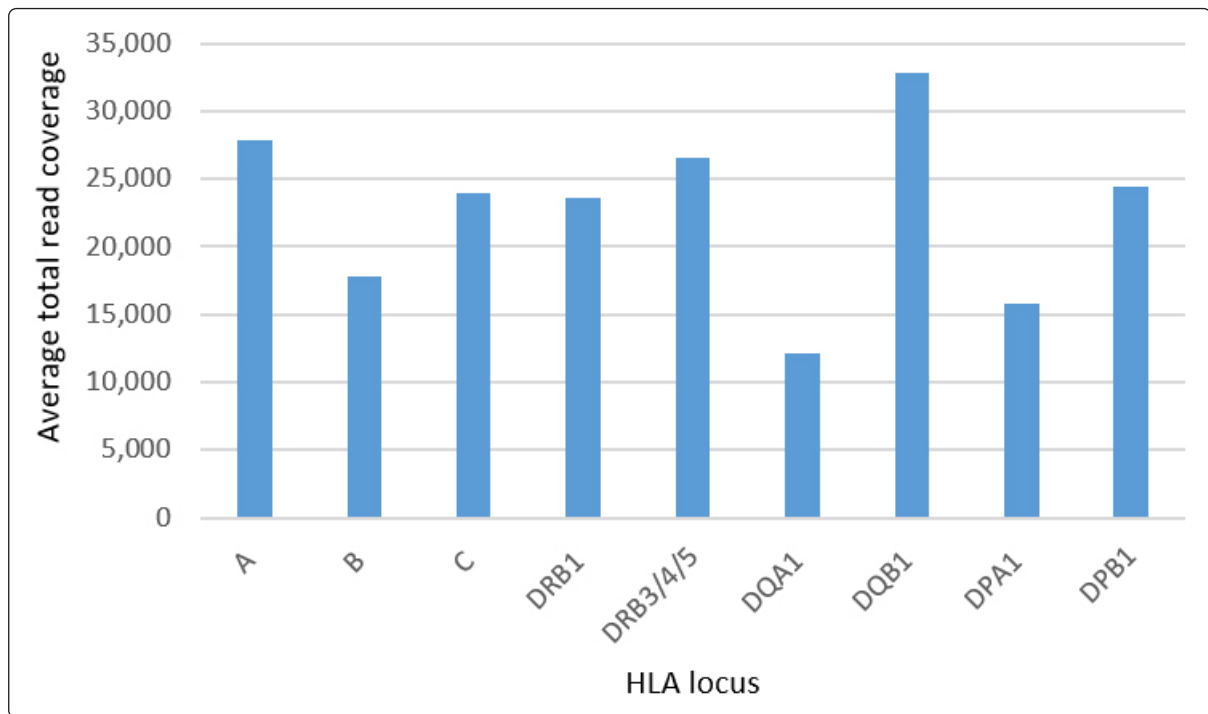


Figure1 Average total read by locus using the HLA TypeStream visual software

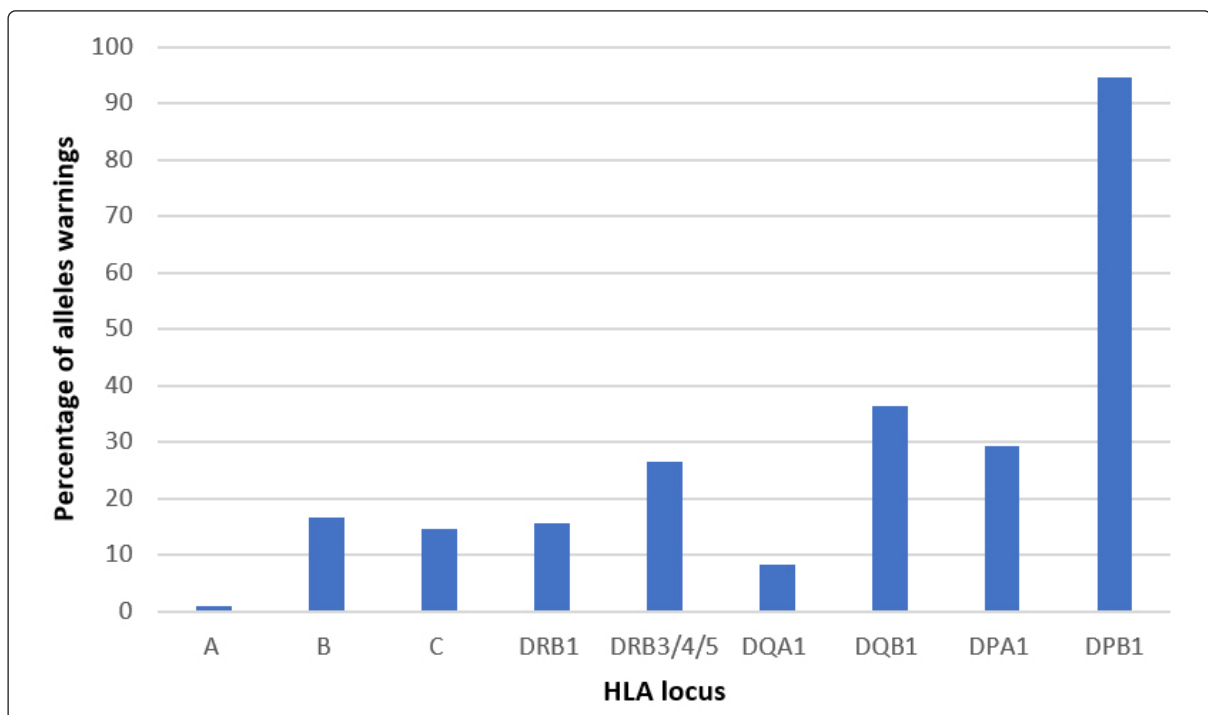


Figure 2 Percentage of alleles warning for each HLA locus analyzed by the TypeStream visual software

reads obtained, there are fewer reads than at the other HLA loci. In this example, NGS detected only the first field, DRB1*04, 15 and DPA1*01, 01, in which proficiency testing results were DRB1*04:03, 15:01, and DPA1*01:03, 01:03, but NGS in the second field cannot be interpreted, which the average total reads were 2,331 and 535, respectively. The DQA1 locus was unreliable with an average total reads of 726, similar to other studies that have shown that the low number of reads is likely to be a problem during the early stages of preparation of gene libraries.^{12,16} Because of HLA high resolution typing of patient and donor organ transplantation may better define the level of matching that can have a devastating impact on graft survival, so the sample that NGS results detected only the first field should be repeated by checking DNA concentrations, individual library concentrations, and the number of reads per sample and locus for confirmation of NGS genotyping.¹⁷ For HLA alleles in different loci, the results obtained from NGS would come out at the high-resolution level from the second-field to the third-field. However, because the results of proficiency testing were obtained in the second-field, they were used for comparison in this study, which were consistent with the results of NGS. There was one sample from the APHIA proficiency testing program that the result was DQA1*01 (new), because some participating proficiency testing laboratories used NGS, the result was reported as DQA1*01:01:01:01 and a novel DQA1*01 allele, where the novel polymorphism occurred in exon 2 codon 79, resulting in a non-synonymous conversion from arginine to cysteine. Moreover, HLA alleles that were not reported in Thai people can still be detected. For example, A*02:291, B*44:12, B*39:25N, B*82:01, and DRB1*11:29 were samples of the UCLA cell exchange program that have different HLA alleles from the Thai population. Based on this study, TypeStream Visual software has shown the warnings in the analysis step.⁸ This means that alleles with warnings were given a positive sign, and if the results were accepted, those results were then selected as the final

results to be used in the reporting. HLA-DPB1*04:01:01 and DPB1*02:01:02 were observed as having the highest percentage of alleles with warnings in this study at the HLA fourth field level which corresponded to the intron of the HLA gene. However, this study compared with proficiency testing at the HLA second field level; therefore, they were not analyzed in this study. In previous study from Thai Red Cross Society, the results of HLA typing were done in HLA-A, -B, -C, -DRB1 and -DOB1 by different NGS platforms, in which AllType™ NGS assay kit showed ambiguity in one sample of HLA-B locus, Holotype HLA (Omixon Ltd., Budapest, Hungary) showed problem in HLA-A locus, and NGSgo (GenDx, Utrecht, Netherlands) showed problem in HLA-DRB1 locus. The results obtained in two fields level which concordance with the known alleles was 99.09%, 99.05%, and 98.18% and times requiring for the whole process of typing varied from 31, 41.50, and 32 hours respectively.¹⁸

The NGS method for HLA typing was used in this study. It took approximately 31 hours for each test of testing and the cost was 12,728 baht per case if 48 samples were performed each time. However, if there were fewer samples at each time, the cost per case would be higher. The cost of NGS is cheaper when compared with other methods such as polymerase chain reaction-sequence specific primer (PCR-SSP), PCR-sequence specific oligonucleotide probe (PCR-SSO), and sequence-based typing (SBT). Moreover, the samples could be detected at high resolution and the problem of ambiguity is greatly reduced. From previous study, NGS has been reported to reduce ambiguity largely arising from heterozygotes or sequencing only the specified regions.^{19,20} A comparison of NGS results by AllType™ NGS assay kit and SBT results for HLA-A and -B alleles showed 100% concordance between the two methods, while there were two discrepant alleles for HLA-C and one discrepant allele for HLA-DRB1.¹² Smith, et al¹⁷ reported a comparison of NGS with PCR-SSO among 289 specimens from five clinical laboratories, in which results were concordant except for PCR-

SSO misassignments in eight specimens and 21 novel sequences uniquely identified by NGS. With few exceptions, PCR-SSO generated ambiguous results while NGS provided unambiguous three-field allele assignments. For complete HLA genotyping of up to 24 samples by either PCR-SSO or NGS, bench work was completed on day one and typing results were available on day two. When compared with this study, the results were available on day three which was longer than the previous studies because of the differences in kit and the machinery platform.

The limitation of NGS is that the equipment used is expensive and requires maintenance costs, and the number of samples inspected each time must be sufficient in order not to increase the cost of the test. To implement in laboratory tests for HLA typing services in organ or hematopoietic stem cell transplantation, the work instruction may need to be changed. For example, at present, if the HLA class I of the donor is identical to that of the recipient, the HLA class II will be tested further. However, with the NGS method used in this study required both HLA class I and II alleles to be examined at the same time. There would be a one-time cost for using the test, but the cost would be cheaper and the results would show more detail, making the selection of donors more suitable.

Conclusion

Evaluation of HLA types by NGS using AllType reagents running on the Ion S5 XL platform showed that HLA-A, B, C, DRB1, DRB3/4/5, DQA1, DOB1, DPA1 and DPB1 were detected. This was concordant with the results of proficiency testing from the three institutions, where results were compared at high resolution at the HLA second field level. Only one sample resulted in HLA at the first field level in HLA class II.

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