HUMAN LEUKOCYTE ANTIGEN (HLA) GENOTYPING BY NEXT-GENERATION SEQUENCING: DEVELOPMENT OF AN IN-HOUSE METHOD AND COMPARISON WITH COMMERCIALLY AVAILABLE METHODS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Accurate genotyping of the human leukocyte antigens (HLA) are crucial for the success of hematopoietic stem cell and solid organ transplantation. Over the past 50 years, numerous methodologies have been used for HLA typing but technological limitations have prevented full interrogation of the HLA gene thus resulting in allelic ambiguity. In order to resolve these ambiguities, additional testing is often required, leading to increased expense and delay in reporting the genotyping results. Recently, advances in nucleic acid sequencing technologies, generally referred to as next-generation sequencing (NGS), have become available. HLA genotyping by NGS is poised to become the new gold standard for several reasons. First, the entire gene can be interrogated, as opposed to a few exons, thus enabling greater resolution of known polymorphisms outside of the T cell recognition site on the HLA molecule. Secondly, missing genetic sequences in the curated databases will become more complete thus improving the algorithms used for alignment to a reference sequence. Lastly, NGS will become the new gold standard because high-resolution genotyping can be achieved without the need for additional time and laboratory resources in order to resolve allelic ambiguities and meet typing requirements required by regulatory agencies. In this thesis I discuss my efforts to employ this new technology by developing an assay for genotyping of the HLA-A, B, C, DRB1, and DQB1 genes. In the new assay, 98% of all alleles typed correctly and unambiguously without the need for any secondary testing. This in-house method is later compared with two recently released commercial kits

and 100% concordance was found between the three methods. Differences in workflow are compared and contrasted. In conclusion, we show that HLA genotyping by NGS produces more correct and unambiguous results than traditional Sanger sequencing without the need for reflex testing.

To my father, Fred, who bought me my first science book.

TABLE OF CONTENTS

LIST OF TABLES

Tables

LIST OF FIGURES

Figures

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use. Dr. Eszter Lázár-Molnár, whose unfailingly positive, can-do attitude joined this project in its latter stages and is now influential in propelling it forward as we now move HLA by NGS into the clinical arena. Finally, I am grateful for my biggest supporter and best friend, my life partner, Val Allyse. She has never doubted in me and has always encouraged me to go father and dig deeper. She has patiently (and sometimes a little less so) listened to my complaints and tolerated my craziness along the way but in the end has unfailingly been there.

CHAPTER 1

HISTORY

The human leukocyte antigen (HLA) system is part of the major histocompatibility complex (MHC), a set of cell surface markers essential for the discrimination of self and foreign antigens. The nature of its discovery is helpful in understanding its biology and function. Three unrelated events were vital in its discovery in the early 1900s. The first was the development and use of inbred mouse strains for scientific studies. An early geneticist, C. C. Little, recognized the value in the use of genetically identical mouse strains and went on to establish Jackson Laboratories, which has since become the world's foremost laboratories for these mice. The second event was interest in tumor transplantation in mice. In 1916, Little and others began experimenting transferring tumors between various strains of mice. They found that tumors were usually rejected by different strains of mice but there was no rejection within inbred strains of mice (Little, 1916). The discovery of blood groups by Paul Ehrlich, J Morgenroth, and Karl Landsteiner (Klein, 1986a) through the use of hemagglutination methods was another critical step. In 1933, J. B. S. Haldane, an English scientist, visited Little and the newly formed Jackson Laboratories and became acquainted with the work of the tumor transfer scientists. He, along with three pairs of inbred mice, returned to England and suggested to his colleague, Peter Gorer, that gene products affecting the reactions to the tumor grafts were similar to the ones detected by the Landsteiner's hemagglutination reactions. Moreover, he suspected these antigens were present in normal tissues and not just in tumor tissues (Klein, 1986a). He left the inbred mice and idea for Gorer to pursue. Using agglutination techniques in mice, Gorer, along with the Jackson Laboratory scientist George Snell, independently discovered the H-2 system, now more broadly known as the MHC (Gorer, 1936, 1937; Klein, 1986b). Eventually, George Snell, along with Baruj Benacerraf, discoverer of MHC

class II, and Jean Dausset, discoverer of the MHC in humans, would receive Noble Prizes in 1980 for their work. Peter Gorer unfortunately died in 1961 but, presumably, would have been included for his ground-breaking efforts.

The discovery of the MHC attracted little attention until Peter Medawar, a physician assigned to work with burn victims during World War II, began studying why skin grafts failed. One of his first patients was a woman who had received two sets of skin grafts from the same donor. The first graft set initially healed but was later rejected but the replacement grafts were destroyed almost immediately. Medawar concluded that the tissue graft rejection is due to an immune response and that the antigens eliciting the reactions were the same as Gorer described. Medawar would go on to receive a Noble Prize in 1960 for this work (Klein, 1986a).

The structural and functional complexity of the HLA molecule would take longer to unravel. An important piece of the puzzle and a new immunological paradigm was uncovered in 1974 by Zinkernagel and Doherty. At that time, it was established that HLA was important for transplantation but the physiological function of the molecule was unknown. Working with lymphocytic choriomeningitis (LCM) virus, Zinkernagel and Doherty discovered that lysis of the LCM-infected cell cultures only occurred when the target cell and the sensitized T cells shared at least one haplotype of H-2 antigens (Zinkernagel & Doherty, 1974a,b). Once again, the use of inbred-strain mice was crucial as it was observed that only some mouse strains were producing virus-specific cytotoxic T lymphocytes (Zinkernagel & Doherty, 1997). This became known as MHC restriction for which the research duo received the 1996 Noble Prize in Medicine. The mechanism behind MHC restriction wasn't understood until crystal structures of the MHC were synthesized

in the late 1980s and revealed the presence of a bound peptide (Bjorkman et al., 1987a; Brown et al., 1988). The bound peptide is generally a self-peptide generated from within the cell but it may also be a foreign peptide from an infectious agent like LCMV. Thus, MHC restriction refers to the dual recognition of the self MHC along with the self or foreign bound peptide (Parham, 2005).

1.1 Biology and Structure

Human MHC genes are found in chromosome 6 and divided into three main regions, class I, class II, and class III, each encoding for structurally and functionally different molecules. The class III region encodes for molecules involved in other immune functions, including complement, and will not be discussed at this time. Overall, the MHC has 421 identifiable gene loci which encode for diverse proteins, most of which are involved in the immune response. In addition to HLA class I, II, and III, the MHC encodes for other important proteins such as olfactory receptors, zinc finger, tumor necrosis factor, and heat shock proteins (Horton et al., 2004). Classes I and II may be further sorted into classical loci important for transplantation due to their high level of polymorphism: Class I includes HLA-A, B, and C. Class II includes HLA-DRB, DQ, and DP (Rodey, 2000). Nonclassical class I and class II molecules are non- or oligo-morphic, and usually do not influence transplantation.

Class I is made up of a transmembrane alpha chain and a soluble beta2 microglobulin chain. The alpha chain forms the peptide binding groove composed of two alpha helices on a beta-pleated sheet platform (Bjorkman et al., 1987b). Together, the HLA and the peptide interact with the T cell receptor. The class I associated peptide is an

endogenously generated molecule that arises from within the cell, thereby providing a form of immune surveillance of the intracellular environment. HLA molecules paired with self-peptides normally do not elicit a T-cell response, but in the case of infection, viral peptides are also presented. In this scenario, cytotoxic T lymphocytes (CTL) recognize the peptide as foreign and destroy or lyse the infected cell (Abbas, 2012).

The class II HLA molecule differs in that it is composed of two transmembrane alpha and beta chains and unlike the class I molecule's ubiquitous presence on all nucleated cells, class II molecules are only found on dendritic cells, activated B cells and a few other specialized antigen-presenting immune cells. The alpha and beta chain join together to form the antigen binding groove (Brown et al., 1993). The class II peptide provides immune surveillance of the extracellular environment. Once displayed on the class II molecule, a T cell will recognize the peptide as foreign but rather than destroying the cell via a cytotoxic immune response, the T cell will interact with other lymphocytes such a B cells to generate antibodies against the foreign antigens (Abbas, 2012).

1.2 Polymorphism

As previously discussed, both class I and II molecules have a peptide-binding groove. Because the HLA molecule must be able to hold a large number of self and foreign peptides, the exons encoding the region around peptide groove are some of the most polymorphic genetics regions (Mungall et al., 2003). This degree of polymorphism is vital for generating the immune response to infection and likely arose as the result of pathogen-driven natural selection (Prugnolle et al., 2005). More specifically, the greatest polymorphism is found in the sites that interact with the self-peptide. This corresponds to

exons 2 and 3 for class I and exon 2 for class II. Traditional HLA genotyping methods, which will be discussed later, have focused on the protein and genetic differences in these exons. To date, over 15,000 HLA alleles have been identified (Robinson et al., 2015) and this number continues to grow rapidly as technological advances in sequencing have led to the interrogation of exons that encode for regions outside the peptide groove. In addition, these same advances now permit genetic sequencing of the intronic regions. It is now estimated that each HLA locus may harbor close to 3.5 million possible rare alleles (Klitz, Hedrick, & Louis, 2012).

Some of the HLA alleles are disease associated. For example, HLA alleles are associated with a number of autoimmune diseases such as celiac disease, narcolepsy, multiple sclerosis, rheumatoid arthritis, Type I diabetes and ankylosing spondylitis (Howell, 2014). Some HLA alleles are associated with increased susceptibilities to infections or may dramatically affect the course of infection (HIV, leprosy, leishmaniasis, hepatitis B, hepatitis C, and human papilloma virus; Karlsson, 2014). More recently, it has been shown that some drugs can bind noncovalently to certain HLA class I alleles and initiate T cell activation (Illing et al., 2012; Ostrov et al., 2012). For example, the presence of HLA-B*57:01 can cause the potentially fatal hypersensitivity response to abacavir. Similarly, HLA-B*15:02 is associated with carbarmazepine hypersensitivity (Profaizer & Eckels, 2012). The FDA now recommends HLA genotyping before patients undergo therapy with either of these drugs (FDA, 2007, 2011).

1.3 Genotyping for HLA

There are numerous indications for performing HLA genotyping such as disease association testing. However, the primary reason for genotyping is to mitigate rejection in solid organ and hematopoietic stem cell transplantation (HSCT). Rejection may occur when recipient T cells recognize either the foreign HLA antigen (direct recognition) or the donor-derived peptides bound to recipient HLA molecule (indirect recognition; Abbas, 2012). Numerous studies have been done showing the benefits of HLA matching (Lee, 2007; Petersdorf et al., 2001; Susal & Opelz, 2013).

HLA genotyping and histocompatibility testing consists of identifying donor and recipient HLA alleles, testing for the presence of preformed HLA antibodies, and finally, performing a crossmatch between the recipient sera and donor cells. Preformed HLA antibodies arise due to previous blood transfusions, pregnancy, or previous transplants. Ideally, all donor organs would be HLA-identical to the recipient but the vast number of HLA allelic specificities and shortage of donor organs makes this all but impossible. Therefore, HLA matching is the avoidance of donor organs that share the same HLA specificity of any antibodies that the recipient has formed. The exception is HSCT in which the donor and recipient HLA type must be nearly identical.

To address the problem of vast HLA polymorphism and many newly discovered alleles, the American Society for Histocompatibility and Immunogenetics (ASHI) created an ad-hoc committee to address the issue of minimal typing requirements. Examining population data, the committee established the Common and Well-Documented (CWD) list of alleles that must be fully resolved (Cano et al., 2007; Mack et al., 2013). Alleles that are “common” have an allele frequency of >0.001 in a reference population of at least 1500

individuals. Well-documented alleles have been observed at least 5 times in unrelated individuals or have been observed 3 times in a specific haplotype (Mack et al., 2013). High resolution genotyping is indicated by a minimum of four digits after the gene name (e.g., HLA-B*35:03), also known as two-field resolution (see Figure 1.1 for explanation of HLA nomenclature). Genotyping for HSCT donors and recipients should consist of minimum of two-field resolution and no CWD allele ambiguities present.

1.4 Complement-Dependent Cytotoxicity (CDC) Assay

There are numerous challenges in HLA genotyping. Individuals inherit one set, haploid genotype (or haplotype), of HLA genes from each parent. HLA alleles are co-dominantly expressed and quite polymorphic, thus, no wild type exists in HLA. The earliest genotyping method was the cytotoxicity test, first developed by Peter Gorer in 1956 (Klein, 1986b). This test, with subsequent modifications, would be the standby test until the advent of molecular methods in the early 1990s. As previously stated, human individuals do not have antibodies to HLA antigens unless they have been previously transfused, transplanted, or been pregnant. Jean Dausset first studied agglutination of erythrocytes in humans using sera from patients who had received multiple blood transfusions (Klein, 1986a). This observation was the first evidence of the human MHC. The discovery of alloantibodies in pregnant women was later observed by Rose Payne (Klein, 1986a; Payne, 1962) and another scientist, Jon van Rood, who proved the antibodies were against the paternal HLA type (Van Rood, Eernisse, & Van Leeuwen, 1958). Maternal allosensitization occurs during delivery as the placenta becomes detached from the uterine wall and small amounts of fetal cells cross into maternal circulation. In

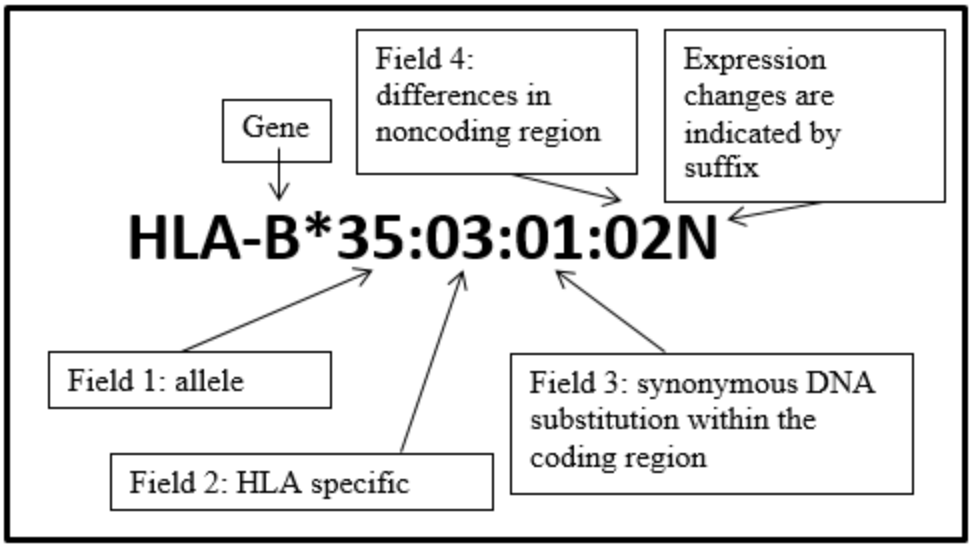

Figure 1.1. HLA Nomenclature. Figure based on information found in Robinson et al. (2015).

turn, the maternal immune system recognizes and forms antibodies against the paternal HLA antigens. Characterization of the sera is important to determine its HLA specificity. The value of the multiparous antisera became quickly recognized and soon trading of sera across the world became commonplace as scientists began hunting for new antigens (Eng & Leffell, 2011).

The complement-dependent cytotoxicity (CDC) test became widespread with the development of the microtoxicity assay by Terasaki (Terasaki & McClelland, 1964). This modification was significant in that only one microliter of antisera was required, thereby reducing the amount of valuable well-characterized antisera needed. Briefly, the assay consists of mixing one microliter of antisera with one microliter of lymphocytes. The lymphocytes are isolated from peripheral blood, lymph nodes, or splenic tissue. After an incubation step, rabbit serum is added to the reaction as a source of complement to increase the sensitivity of the assay. As the HLA antibodies bind to the cell surface, complement is activated and cell death occurs. After another incubation step followed by the addition of a supravital stain such as ethidium bromide or carboxymethylfluorescein, the cells are assessed microscopically for cell death. When more than 25% of the tested cells are dead, the cells are considered to share the complementary HLA antigen (Rodey, 2000).

The CDC assay is relatively easy and quick to perform but numerous shortcomings limit its usefulness (see Table 1.1). As previously mentioned, the antisera must be well-characterized in how well it will react with the multitude of HLA antigens. Several antigens share common epitopes, often called public epitopes, and it is a common occurrence for antibodies to be formed against these cross-reactive groups thus forming public antibodies (Rodey, 2000). Therefore, a single antibody can react with a number of related HLA

Table 1.1. Advantages and Disadvantages of the CDC Assay.

Complement-Dependent Cytotoxicity Assay
Advantages • Relatively quick and easy to perform. • Can determine if null antigens are present if family studies are available.
Disadvantages • Need well-characterized antisera. Antibodies need to be specific for a few HLA antigens. • Requires a large number of viable lymphocytes. • Complement must not have too much lytic potential. Cell death should only occur in cells with corresponding HLA specificity. • Antisera generally only against broad specificities; allele-specific antibodies are rare and difficult to find. • Interpretation of cell death is subjective. • Cannot determine if HLA antigen is homozygous unless family studies are conducted. • Loci with low HLA expression can be mistyped.

antigens and care must be taken to use a panel of antisera that contains a variety of public and private specificities to aid in interpretation. The source and lot of complement is another area for concern. Rabbit serum has traditionally been used as a source of complement, but typically individual lots of commercially available sera vary in reactivity. Therefore, careful titration experiments must be performed before purchase to ensure that the serum is not overly reactive. Rabbit sera (and other mammalian sera) contain heterophilic antibodies that will react to all human cells (Rodey, 2000) and a high titer of heterophilic antibodies can mask the effect of the antisera and obscure the true HLA genotype by indiscriminately killing cells. For this reason, plus the requirement by the National Marrow Donor Program (NMDP) for high-resolution genotyping and the commercial availability of DNA genotyping kits, genotyping by CDC has largely fallen out of favor.

1.5 DNA Methods

The lack of reproducibility and the need for the variable biological reagents such as antisera and complement place serological assays at a disadvantage. The first DNA-based assay for HLA typing was the restriction fragment length polymorphism (RFLP) assay (Le Gall et al., 1986) but was difficult to perform and prone to technical difficulties (Rodey, 2000). The advent of the polymerase chain reaction in 1987 (Mullis & Faloona, 1987) revolutionized the field and, for the first time, a more reproducible and standardized method for HLA genotyping was available. The main PCR-based assays used for HLA genotyping are sequence-specific primers (SSP), sequence-specific oligonucleotide probe (SSOP) hybridization, and the Sanger sequence-based typing (SBT) assays. The Sanger

SBT method will be discussed in a separate section.

The sequence-specific primer (SSP) assay is the most simple of the molecular assays. Briefly, primers are designed to hybridize to only one HLA allele or to a group of similar alleles (Olerup & Zetterquist, 1991). Following completion of PCR, the individual reaction products are added to an agarose gel and electrophoresis is performed. The larger sized amplicons move more slowly through the gel as opposed to shorter sized ones. Therefore, HLA alleles can be assigned based on the presence of the correct sized band. This assay is easy to perform but can be tedious due to the large number of individual reactions that must be prepared. Furthermore, it is difficult to perform on more than a few samples at any one time thereby limiting its usage to smaller labs.

The sequence-specific oligonucleotide probe (SSOP) hybridization assay is better for allelic resolution because rather than using individual primers to isolate specific alleles, entire exons are amplified by generic primers (Table 1.2). Following PCR, biotin-labeled probes are hybridized to the regions of interest. Following a wash step to remove any unbound probes, the samples are labeled with a chemoluminescent substrate that has been conjugated to a streptavidin molecule (Erlich, Opelz, & Hansen, 2001). There are a number of modifications to this protocol depending upon the manufacturer. For example, in early SSOP assays, the probes were physically bound to a nylon or nitrocellulose membrane (Saiki, Walsh, Levenson, & Erlich, 1989). More recently, the use of color-coded microspheres coated with individual DNA probes for use on the Luminex instrument have gained popularity for their ease of use and scalability (Dunbar, 2006). In this modification, the probes are labeled with biotin and the microspheres have replaced the nylon or methylcellulose membrane (Itoh et al., 2005). The Luminex instrument uses two fixed

Table 1.2. Advantages and Disadvantages of SSP and SSOP Assays.

PCR-Based Assays: SSP and SSOP
Advantages • Chemically synthesized reagents improve standardization and reproducibility. • Smaller amounts of sample required. Can be from any source where DNA can be extracted. • More complete genotyping assignment based on nucleic acid sequence, not protein sequence. • Increased sensitivity and specificity. • SSOP suitable for large numbers of samples.
Disadvantages • Time consuming. • Expensive laboratory equipment may be required. • Low allelic resolution and thus more ambiguities. • Only exons 2 and 3 are interrogated.

wavelength lasers, one to characterize the microsphere, based on its pattern of color fluorescence, and the other laser measures the amount of fluorescent antibody, or bound probe, on the surface of the microsphere (Dunbar, 2006).

The Luminex SSOP assay is well-suited for handling large numbers of samples. While theoretically the number of probes is limitless, there is a physical limitation to the amount of spectral separation between the beads. The original Luminex instrument could distinguish between 100 individual microspheres, but recently Luminex introduced the Flexmap 3D, which can discriminate between 500 uniquely labeled microspheres (Luminex, n.d.). Regardless, the vast polymorphism of HLA greatly exceeds the capabilities of SSO.

1.6 Sanger Sequencing

Sequence-based typing (SBT) became a possibility in 1977 when Fred Sanger and colleagues developed a method to determine actual genetic sequences. It is based on the principle of using chain-terminating dideoxynucleotides (ddNTP) to effectively halt the addition of nucleotides by the polymerase. The ddNTP is a deoxynucleotide that has been chemically altered to lack the 3'-OH group required for the addition of the next nucleotide via formation of a phosphodiester bond (Sanger, Nicklen, & Coulson, 1977). Initially, nucleotide labeling was done with 32P but was later replaced with a fluorescent dye, making the assay less hazardous and more economical (Smith et al., 1986). In addition to the four ddNTPs, the reaction master mix contains a mix of unaltered deoxynucleosidetriphosphates (dNTPs). The reaction proceeds as incoming dNTPs are incorporated into the growing DNA chain. Each time a ddNTP is incorporated, the chain

ends and the fragment is labeled with a fluorescent tag unique to the individual nucleotide incorporated. After sequencing, the products are size separated using capillary electrophoresis. Shorter fragments migrate faster. The sequence is then read from the bottom to the top of the gel (Buckingham, 2012). Development of automated sequencers which use raster scanning lasers to capture the fluorescent wavelengths of the individual fragments have reduced the need for the manual reading of gels and improved the scalability of the method. These improvements have decreased errors and increased the run-throughput of the assay (Mardis, 2013).

Sanger sequencing for HLA typing is considered to be the gold standard for obtaining high resolution genotyping. Typically, SSP or SSOP methods do not achieve typing resolution that is free of CWD ambiguities. As previously discussed, the number of HLA alleles continues to grow as more individuals and regions outside of exons 2 and 3 are sequenced. Furthermore, it is economically and technologically tenuous to resolve every allele using either SSP or SSOP. Fortunately, Sanger sequencing is able to resolve several CWD ambiguities and has been the method of choice for genotyping donors and recipients for HSCT. Nevertheless, there are some shortcomings (Table 1.3).

One of the major drawbacks to Sanger sequencing is that it is inherently qualitative. While the size of the electropherogram peak is representative of the number of copies of a nucleotide observed, it is semiquantitative at best (see Figure 1.2; Voelkerding, Dames, & Durtschi, 2010).

Read-length or the length of the actual DNA sequence is limited to less than 1000 bp due to physical limitations of the method, including matrix of the polyacrylamide gel or capillary, or electrophoresis conditions preventing longer reads from being sequenced

Table 1.3. Advantages and Disadvantages of Sanger Sequencing for HLA Genotyping.

Sanger Sequencing
Advantages • Numerous commercial kits available. • Widespread usage and experience. • High-resolution HLA genotyping.
Disadvantages • Cannot resolve cis/trans ambiguities. • Only exons 2 and 3 are interrogated by the commercial kits. • Expensive. • Time-consuming. • Qualitative. • Read length is generally 300-1000 bp.

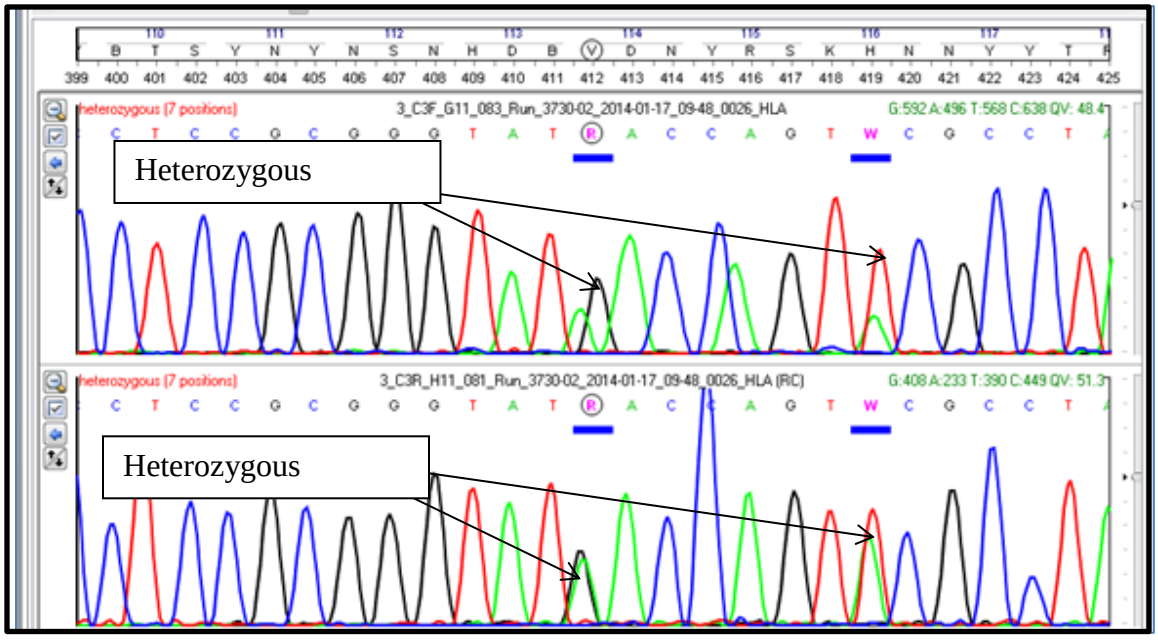

Figure 1.2. Sanger Electropherogram. Each base is color-coded: C = blue, G = black, A = green, T = red. One individual peak indicates the sequence is homozygous at that position. A heterozygous position is indicated by the presence of two peaks. In this example, the first heterozygous position shows overlapping G and A peaks. The second heterozygous position shows overlapping T and A peaks. Both complementary strands of DNA are sequenced (forward and reverse) as a practice to confirm presence of variants.

(Mardis, 2013).

There are three main causes of allelic ambiguities in SBT (Voorter, Mulkers, Liebelt, Sleyster, & van den Berg-Loonen, 2007). The first is when a polymorphism defining two alleles is outside the region sequenced. Traditionally, only exons 2-3 are sequenced for class I, and only exon 2 is sequenced for class II. A well-known example of this issue is DRB1*14:01 versus DRB1*14:54. Both share the same sequence for exon 2 but differ by one base in exon 3. Since only exon 2 is sequenced, DRB1*14:54 samples are typed and reported as DRB1*14:01. This is significant because allele frequency studies have shown that DRB1*14:54 is most often the correct allele because it has a much higher allele frequency (Xiao et al., 2009).

The second cause is due to the lack of fully characterized HLA sequences in the international ImMunoGeneTics (IMGT) database. It is estimated that less than 10% of the alleles have been completely sequenced from 3' untranslated region to 5' untranslated region (Lind et al., 2013). It is very likely that as alleles are fully sequenced, more alleles will be discovered.

Cis/trans or phase ambiguities are the third type of HLA ambiguity. These ambiguities may exist within a single exon or between exons. In SBT, both heterozygous alleles are amplified and sequenced together. This can lead to multiple allele combinations that share the same base. Figure 1.3 is a simple representation of two chromosomes that are alike with the exception of the second polymorphic base. Here, it is impossible to determine if the "T" variant is associated with either "G" or "A."

The Sanger electropherogram would display this scenario the same it would with any other heterozygous position as demonstrated in Figure 1.2. In that example, the first

heterozygous position is a G (guanine) and an A (adenine) and the second heterozygous position is C (cytosine) and A (adenine), however, it is impossible to determine from this sequence the correct pairing of these bases. The first nucleotide G could pair with either A or C, and conversely A could pair with either A or C. Only by comparing the entire sequence against a reference sequence can the correct allele pair be determined. Figure 1.4 is an example of a typical situation seen in HLA typing. Here, it is difficult to determine if the correct B locus pair is B*15:01, 35:01 or B*15:20, 35:43 because B*15:01 and B*15:20 share the same sequence for exon 2 and B*15:01 and B*35:43 share the same sequence for exon 3. Since exons 2 and 3 are amplified and sequenced separately, resolution of these ambiguities is impossible. Both B*15:20 or 35:43 are less frequently observed alleles, however, they still meet the criteria for inclusion on the CWD list and must be resolved. Resolution can be achieved by one of two methods. The first is to use a group-specific sequencing primer to only amplify one allele group via SBT (see Figure 1.5).

In this example, using a sequencing primer specific to exon 3 and B*08:01 (see arrow) would rule out the possibility of the B*07:05, 08:07 pair. The other method to establish the correct allele pair is to sequence a fragment long enough to span polymorphisms in both exons. This will be discussed further in the next section. Alternatively, resolution of CWD alleles may be done by genotyping the entire family.

1.7 Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS) is the latest method applied for HLA typing and is poised to replace Sanger sequencing as the new gold standard. NGS is a broad term used to categorize advanced DNA sequencing platforms. NGS is also called massively parallel

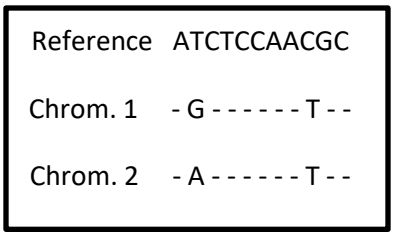

Figure 1.3. Cis/Trans Ambiguity Example.

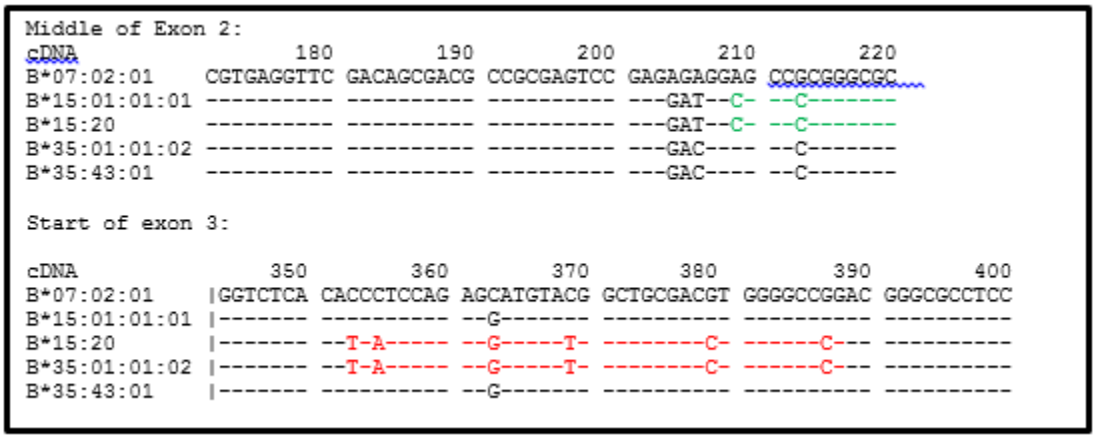

Figure 1.4. Phasing in HLA.

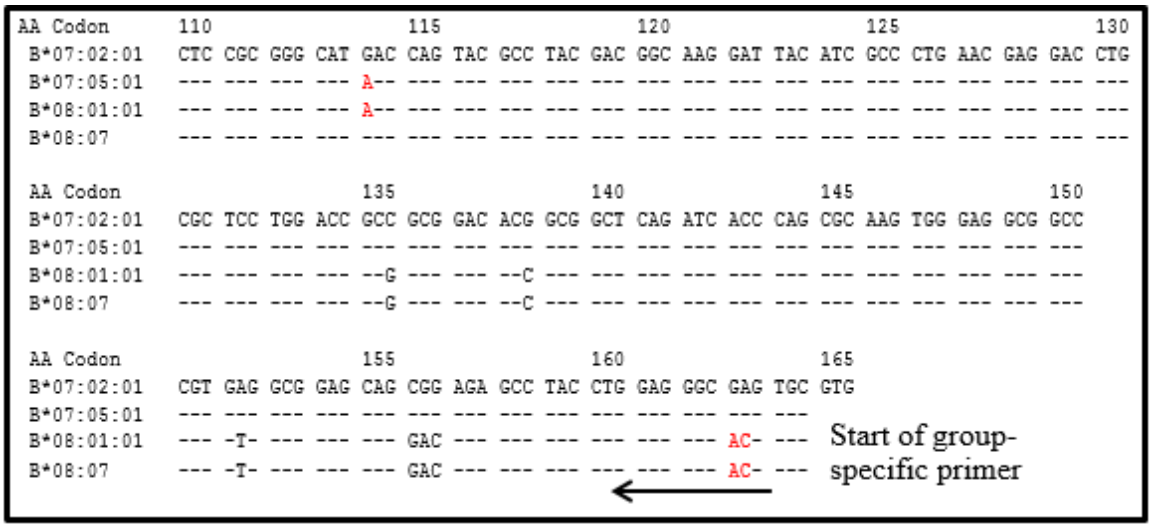

Figure 1.5. Group-Specific Primers to Resolve Ambiguities in Sanger Sequencing.

sequencing because each fragment is individually amplified and sequenced in a digital fashion (Mardis, 2013). This massively parallel manner is what makes NGS quantitative. There are several NGS platforms available, however, only Illumina's proprietary technology, sequencing by synthesis, will be discussed in this thesis. The Illumina MiSeq was selected as our instrument of choice because of its low error rate in comparison to other platforms (De Santis et al., 2013). The general steps for HLA NGS are listed in Table 1.4.

The process starts with long-range PCR. For HLA-A, B, and C, the entire gene is sequenced. DRB1 has a large intron 1 making long range PCR difficult; therefore, only exons 2 through 4 are amplified. After amplification, construction of the DNA library begins with the shearing of the large amplicon to create random, overlapping DNA fragments of varying lengths. Two main methods are used to break up the large DNA amplicons: sonication or enzymes (Voelkerding et al., 2010). The resulting fragments have large overhangs and require repair work on both the 3' and 5' ends to either blunt or fill in the DNA strands as needed. Monoadenylation of the 3' end prepares the fragment for the addition of a synthetic adaptor that is specific to the DNA oligo on the Illumina flow cell (Mardis, 2013). Molecular barcodes, unique to each individual sample, are added at this stage, enabling the pooling of multiple samples together. This feature facilitates more efficient usage of the high-throughput capabilities of the Illumina sequencer. After a series of clean-up steps, the final DNA library is quantified and adjusted to the correct concentration in preparation for cluster generation and sequencing by synthesis on the Illumina MiSeq.

The massively parallel feature of NGS occurs during cluster generation. After the

Table 1.4. General Steps Required for HLA Genotyping by NGS.

General Steps Required for HLA Genotyping by NGS
Long-range PCR
Library Construction • Quantification of amplicons • Fragmentation • End-repair and monoadenylation • Ligation of Illumina-specific adaptors • Ligation of unique molecular bar code to each sample • Size selection • Clean-up • Final pooling and quantification
Cluster generation on the Illumina MiSeq
Sequencing by synthesis on the Illumina MiSeq
Analysis

pooled library has been added to the MiSeq Reagent cartridge, the DNA libraries individually hybridize to oligos on the flow cell (see Figure 1.6). The Illumina flow cell consists of a glass slide upon which oligos, complementary to the DNA library adaptors, have been covalently attached (Metzker, 2010). Once bound, the individual fragments are amplified using isothermal bridge amplification in order to create clusters of cloned fragments (Voelkerding et al., 2010). The oligos are spatially separated so that any DNA library that attaches remains at that physical location during the entire sequencing process (Shendure & Ji, 2008).

After bridge amplification, the clusters are ready for sequencing. The DNA library adaptors also contain genetic regions that are complementary to the primers required for initiation of sequencing. Sanger sequencing relies on the use of altered nucleotides which lack the 3' -OH group. Incorporation of the dideoxynucleotides stops the reaction. In contrast, the Illumina method makes use of reversible terminators. The nucleotides contain an identifying fluorescent dye and a unique blocking group at the 3' -OH position (Bentley et al., 2008). After incorporation of the complementary altered nucleotide to the cluster template, the remaining nucleotides are washed away. An image is taken to determine the identity of the new nucleotide before the addition of tris(2-carboxyethyl) phosphine (TCEP) to remove the fluorescent moiety and regenerate the 3'-OH group in order to prepare for the addition of the next cycle of nucleotides (Bentley et al., 2008; Metzker, 2010). A distinguishing feature of massively parallel sequencing is that the sequencing reaction is executed in a nucleotide-by-nucleotide manner in contrast to the detached separation and detection of previously sequenced Sanger reaction products (Mardis, 2011).

After completion of sequencing, the multiple are aligned to a reference gene and

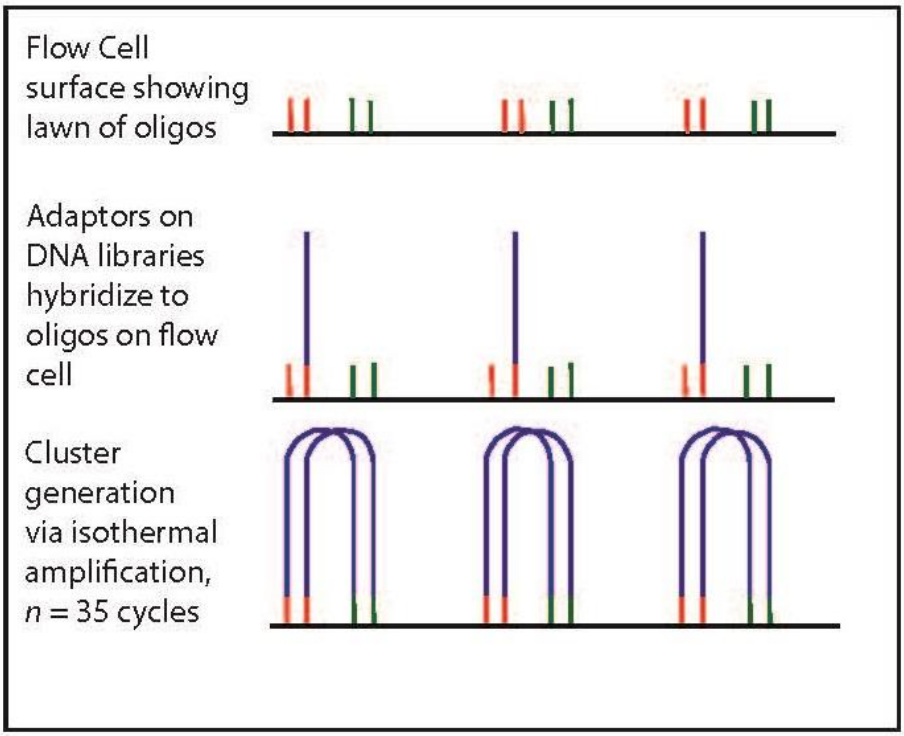

Figure 1.6. Cluster Generation by Bridge Amplification.

can be “tiled’ across the length of the gene (see Figure 1.7).

1.8 HLA Genotyping by NGS: The New Gold Standard

HLA genotyping by NGS will become the new gold standard for the following reasons. First, the entire gene is interrogated as opposed to a few exons. This permits greater resolution of known polymorphisms outside of exon 2. For example, distinguishing between DRB1*14:01 and DRB1*14:54 is not an issue with NGS, since exon 3 will be sequenced. However, the lack of full sequences in the IMGT Database remains a large, but not insurmountable, problem as the expense of whole gene sequencing is becoming cost effective with NGS. Eventually, the missing genetic sequences in the IMGT database will be completed. One outstanding question about high-resolution genotyping is that it may not affect clinical outcomes. A recent study involving a small number of HSCT patients who had their HLA genotyping performed by NGS, found that alleles with exonic mismatches outside the antigen recognition site were not shown to affect transplant outcomes (Hou et al., 2016). Further retrospective studies with larger numbers of patients will be required to definitively answer this question. Regardless, the final reason NGS will become the new gold standard is because high-resolution genotyping can be achieved easily without the need for additional time and laboratory resources to resolve CWD alleles or to meet minimal NMDP typing requirements. Even with Sanger sequencing, it is not uncommon for additional testing to be done in order to satisfy these requirements. This leads to the delayed reporting of results and requires that additional reagents such as group-specific primers be kept on hand. The additional reagents require the same quality control measures and proficiency testing as required by normal laboratory procedures. High-

resolution genotyping is achieved by a combination of NGS-specific approaches: quantification of cloned fragments, phasing of the entire gene, and paired end sequencing. Examples of quantified fragments and phasing are shown in Figure 1.7.

In Figure 1.7, the alignment of fragments against HLA-A for the last part of intron 1 through exon 3 are displayed. The vertical colored bars in each read represent bases that are not in agreement with the reference sequence. The pink and yellow reads represent forward and reverse strand reads. Through random fragmentation and the clonal amplification of individual fragments, over-lapping and multiple copies can be aligned to the reference allele across the gene. It is through this strategy the entire gene has the potential to be fully linked or phased. As the process is quantitative, the number of times a nucleotide base is sequenced is known as the depth of coverage (Voelkerding et al., 2010). This is of particular importance in HLA where individuals are typically heterozygous and have numerous polymorphisms. Unfortunately, errors in sequencing do occur and it is impossible to distinguish an error from a polymorphism, therefore, having a sufficient depth of coverage is important to determine the true base call (Sims, Sudbery, Ilott, Heger, & Ponting, 2014). Figure 1.8 shows an example of a sequencing error. Arrows point to two positions that are not in agreement with the reference sequence.

Since most individuals are heterozygous, the minimum and average depths of coverage are crucial quality metrics to ensure that both alleles are balanced in the number of times that they are sequenced. For example, each heterozygous allele should each have 50% of the total reads (Voelkerding et al., 2010).

The correct size distribution of the DNA fragments is crucial for phasing. Fragments too short may lead to gaps within the sequence, especially in areas where

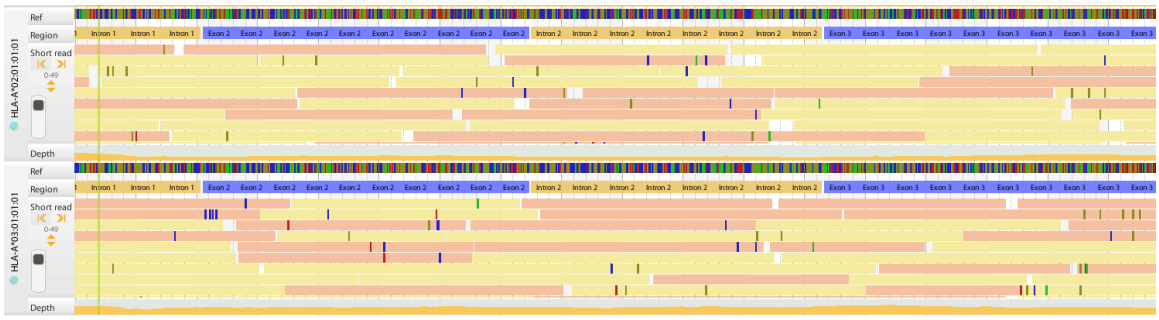

Figure 1.7. Example of Tiled Fragments Aligned Against a Reference Gene in NGS.

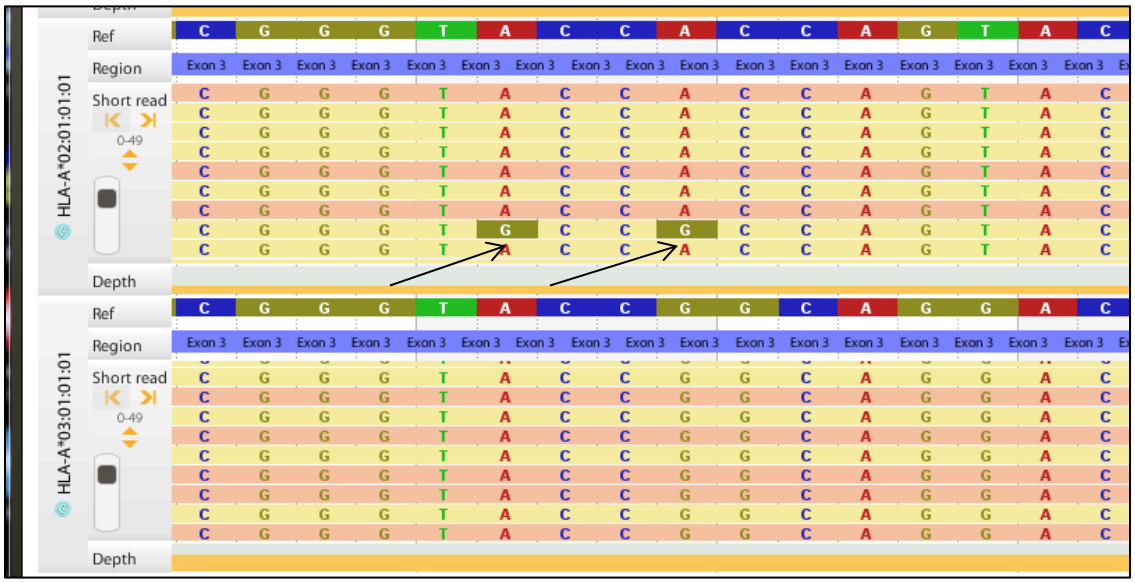

Figure 1.8. Example of Sequencing Error in NGS.

there are many repetitive elements (Voelkerding et al., 2010) but long fragments are more difficult to create and size select in the library construction process. Furthermore, the signal-to-noise ratio limits both the fragment size and read-length for the Illumina sequencers (Mardis, 2013). The ideal fragment size is long enough to span and link polymorphism across exons and introns in order to eliminate cis/trans ambiguities. In tandem with fragment size, the reagent kit read-length must be considered. Each Illumina reagent kit contains a limited amount of reagents to produce a set number of cycles or number of times an individual nucleotide is added to the DNA template. In the work that follows, 300, 500, and 600 bp cycle (or read-length) kits are evaluated and compared. Using a 600 bp read-length kit with fragments less than 200 bp is an inefficient use of the kit and its reagents. Therefore, identifying the intersection of ideal fragment size with read-length is important for complete phasing. Finally, paired-end sequencing is a unique feature of NGS and serves to improve overall sequencing confidence because each cluster is sequenced from both ends, thus creating two reads for one fragment (Coonrod, Durtschi, Margraf, & Voelkerding, 2013). Through the unique features of NGS, high-resolution genotyping can be achieved in just one assay as opposed to several assays as often, currently required. Table 1.5 lists the advantages and disadvantages of Illumina-based NGS.

1.9 Specific Aims of Research

Based on its success in genetic testing, NGS technology has the potential to resolve long standing problems associated with HLA genotyping, therefore we hypothesized that HLA genotyping by NGS is a cost-effective method to produce more correct and

Table 1.5. Advantages and Disadvantages of NGS (Illumina).

NGS Sequencing
Advantages • Interrogates the entire gene (with some exceptions). • Elimination of cis/trans ambiguities (phasing). • Quantitative.
Disadvantages • Requires large capital equipment purchases. • Short read length (≤ 600 bp). • Significant hands-on time required for library preparation (without automation).

unambiguous results than Sanger sequencing without the need for reflex testing. This project arose before commercial kits became available and there were several unanswered questions. Using off-the -shelf reagents, we sought to determine if we could obtain accurate HLA genotyping on 10 well-characterized samples from the International Histocompatibility Working Group (IHWG) using the Illumina MiSeq.

1. One of the most important questions was if all 5 loci (HLA-A, B, C, DRB1, and DQB1) could be indexed for the same sample (index-by-patient) or if each amplicon required individual indexing (index-by-amplicon). HLA loci are homologous due to their origin via gene duplication events (Parham, 1999). We sought to know if this homology would impede accurate genotyping assignments particularly in samples indexed as a whole as opposed to individually indexing each locus. This is critical for clinical testing, as indexing-by-amplicon is far more costly than indexing-by-patient.

2. Part of ensuring that phasing is made across the entire gene, the ideal fragment size of the DNA library must be determined. Ideally, DNA fragments are long enough to span distant polymorphisms in order to resolve cis/trans ambiguities. Fragments too short will prevent phasing but fragments too long will interfere with cluster generation. We begin with a simple comparison of two fragment sizes, 100-300 bp and 300-600 bp. These same libraries were compared using two different read-length MiSeq reagent kits, 2x150 and 2x250. Ten IHWG samples were compared to determine best indexing strategy, fragment length size, and read-length kit. Figure 1.9 is a flowchart of our comparisons. The experiments used to answer these questions and the subsequent results can be found in Chapter 2.

3. Chapter 3 builds on the work shown in Chapter 2 (see Figure 1.10). Here,

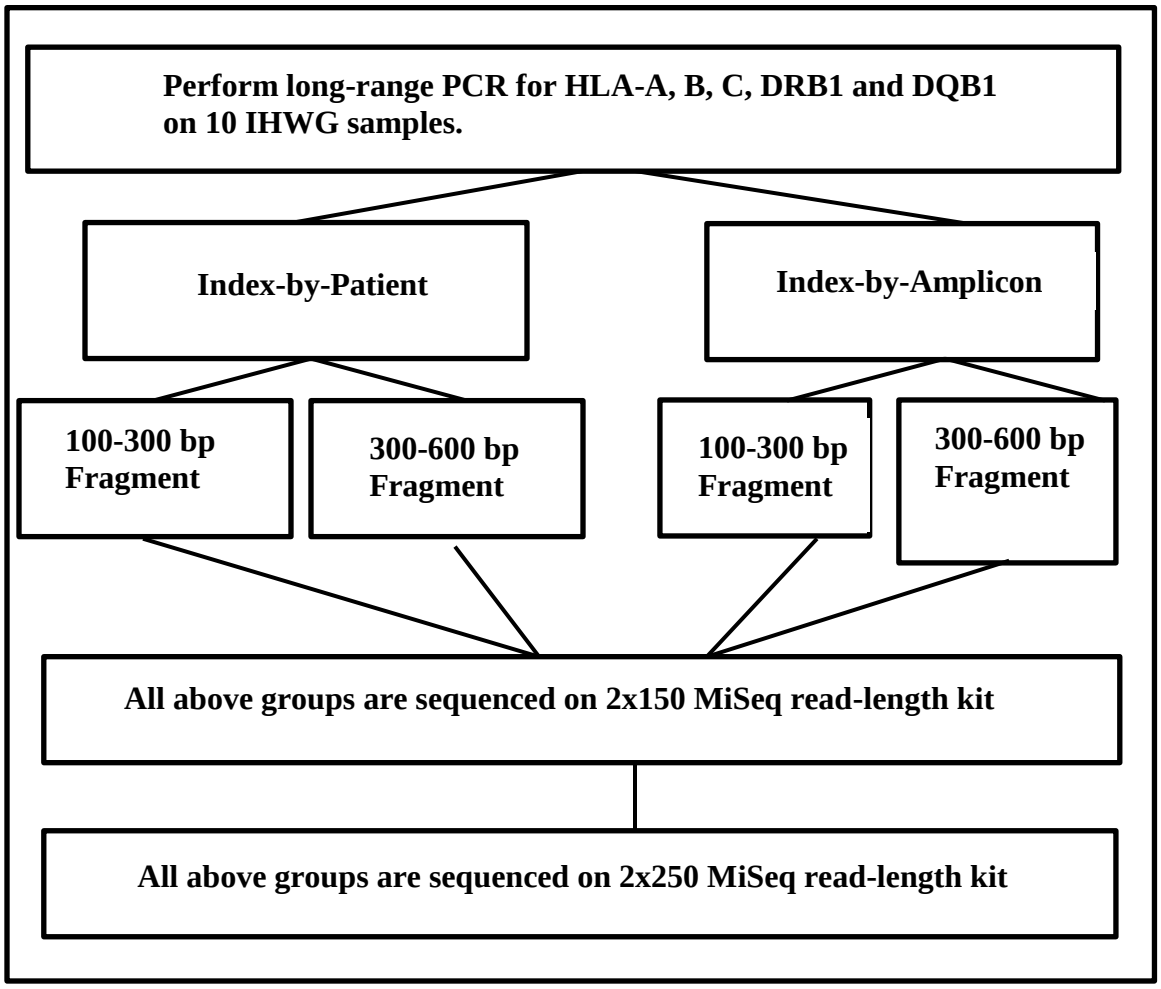

Figure 1.9. Flowchart of Chapter 2 Experiments.

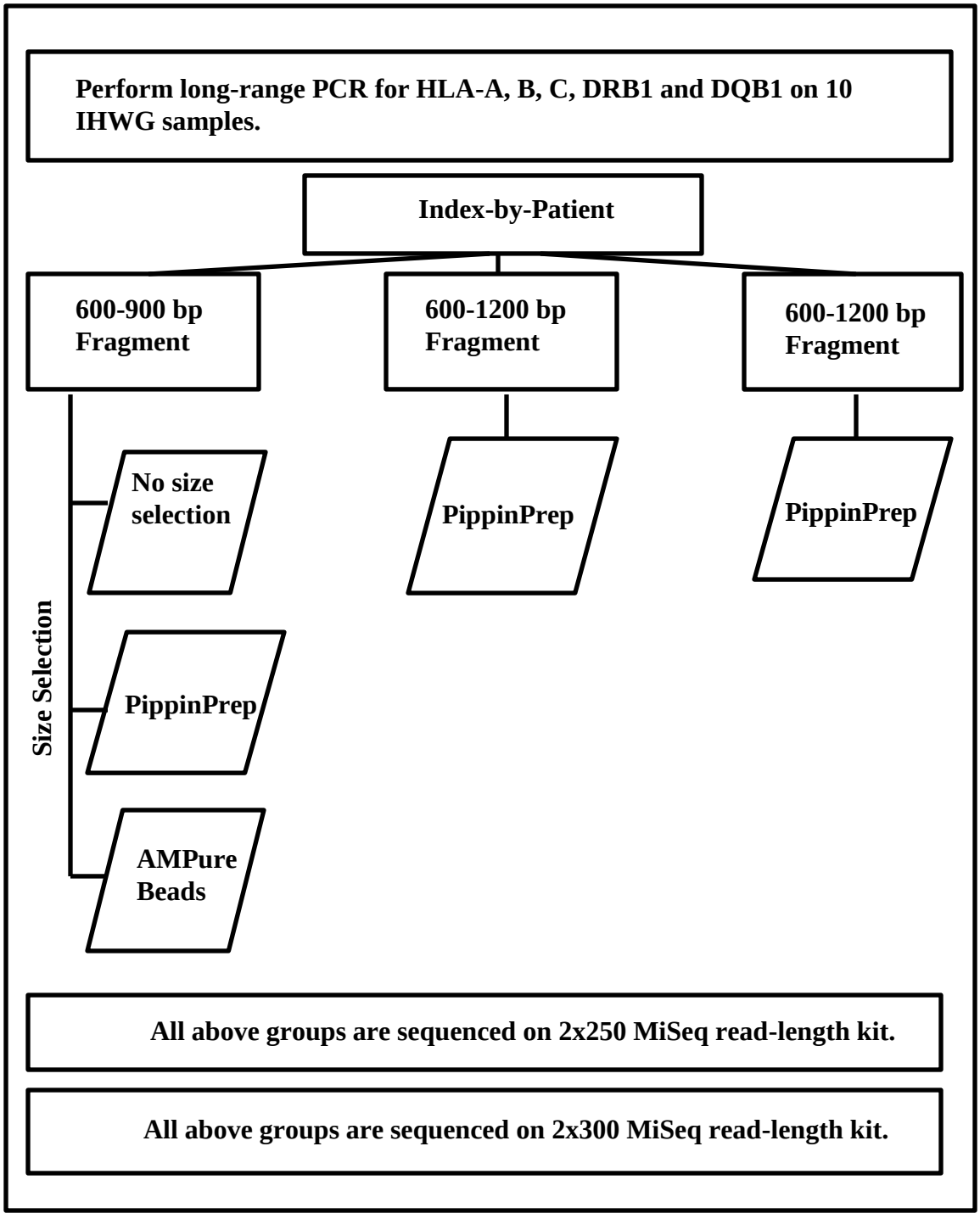

Figure 1.10. Flowchart of Chapter 3 Experiments to Determine Optimal Fragment Size and Read-Length Kit for the In-House Method.

fragment sizes are expanded up to 1200 bp and an additional MiSeq read-length kit is evaluated, 2x300 bp. Early on, it was observed that the AMPure beads could not size-select libraries greater than 900 bp. Therefore, three size-selection strategies were investigated and compared for the 600-900 bp fragment groups: no-size selection, AMPure beads, and an automated gel electrophoresis method—the Sage Science PippinPrep. The same 10 IHWG samples are used throughout. From this work, we establish the optimal conditions for performing the in-house HLA NGS assay.

4. Finally, two newly available commercial kits, the Omixon Holotype X2 24/7, and the Illumina HLA TruSight, are evaluated and compared against the optimized in-house conditions. This is done by using 38 deidentified clinical samples from the Histocompatibility and Immunogenetics Laboratory at the University of Utah. These samples have been HLA typed by Sanger sequencing and consist of DNA from hematopoietic stem cell transplant donors and recipients. This comparison will examine over-all typing accuracy, allele-resolution, and ease of work-flow. Chapter 4 is the conclusion of this research.

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CHAPTER 2

REPORT ON THE EFFECTS OF FRAGMENT SIZE, INDEXING, AND READ LENGTH ON HLA SEQUENCING ON THE ILLUMINA MISEQ

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Report on the effects of fragment size, indexing, and read length on HLA sequencing on the Illumina MiSeq

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Next-generation sequencing

ABSTRACT

Single-molecule sequencing should allow for unambiguous, accurate, and high-throughput HLA typing. In this proof of principle study, we investigated the effects of fragment size for library preparation, indexing strategy, and read length on HLA typing. Whole gene amplicons of HLA-A, B, C, DRB1, and DQB1 were obtained by long-range PCR. For library preparation, two fragment sizes were evaluated: 100–300 bp and 300–600 bp. For sample multiplexing, two indexing strategies were compared: indexing-by-amplicon, where each individual amplicon is barcoded, and indexing-by-patient, where each patient's five loci are equimolarly pooled after PCR and indexed with the same barcode. Sequencing was performed on an Illumina MiSeq instrument using paired-end 150 bp and 250 bp read lengths. Our results revealed that the 300–600 bp fragments in the 2 × 250 MiSeq group gave the most accurate sequencing results. There was no difference in HLA typing results between the two indexing strategies, suggesting that indexing-by-patient, which is much simpler, is a viable option. In conclusion, enzymatic fragmentation of pooled whole gene amplicons is a suitable strategy for HLA typing by next-generation sequencing on the Illumina MiSeq.

1. Introduction

Current HLA typing techniques include sequence-specific oligonucleotide (SSO) methods and Sanger sequencing. While SSO methods are generally suitable for solid organ transplantation, hematopoietic stem cell transplantation (HSCT) requires Sanger sequencing for allele-level resolution and matching. HLA typing by SSO methods and Sanger sequencing have many challenges. First is the vast polymorphism of more than 11,000 unique HLA alleles [1], making it difficult to fully characterize every allele. Second is the failure of current methods to distinguish between cis and trans nucleotide substitutions, often leading to family studies in order to differentiate between each parentally-inherited HLA allele or require laborious cloning-based approaches. Third, is the fact that less than 10% of the known alleles have been completely sequenced from beginning to end, thereby producing ambiguous HLA typing results. The typing resolution required by the National Marrow Donor Program [2] to meet the minimum allele requirement as defined by the common and well-documented (CWD) HLA allele list [3] also creates a high burden of reflexive testing between SSO methods and Sanger sequencing.

Recent advances in next generation sequencing (NGS) suggest that unambiguous, accurate, and high-throughput HLA typing may now be in the realm of technological and economic possibility [4]. NGS can meet the challenges of HLA typing because it is single-molecule sequencing that provides both quantitative and qualitative typing data. For example, sequencing data obtained by Illumina NGS is generated from clonal clusters of the same library fragment on a single flow cell. Since each clone is individually sequenced, parentally inherited alleles can be easily separated and identified [5]. This quantitative sequence information along with full gene interrogation provides far more information than traditional HLA typing methods [6].

Early studies on HLA typing by NGS used specific primers to amplify and sequence the desired exons [7]. Since the power of NGS lies in its ability to interrogate a large genetic region that includes all exons and introns, thus gaining more information and less ambiguity, whole gene testing is preferred. Sequencing whole genes is more difficult than sequencing short exons. Whole-gene testing requires long-range PCR and PCR efficiency decreases with increasing amplicon length. Moreover, the large

Abbreviations: NGS, next-generation sequencing; HSCT, hematopoietic stem cell transplantation; CWD, common and well-documented; SSO, sequence-specific oligonucleotide.

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amplicons must be first fragmented for most current NGS sequencing technologies. Other potential difficulties are, however, similar to that of exon-based PCR and include co-amplification of pseudogenes or non-classical HLA genes [8] that may make the analysis more difficult with the potential for preferential amplification of alleles [9].

Following DNA amplification, amplicons must be prepared for DNA sequencing. This process, also known as library preparation, consists of fragmenting the long-range PCR products, repairing terminal ends, ligating adaptors, and adding barcodes. Fragmentation leaves terminal end overhangs of varying lengths on either the 5′ or 3′ side of the DNA. In order for efficient ligation to occur, the overhangs must be trimmed, phosphorylated and a 3′ adenine added [10,11]. After end-repair, synthetic oligonucleotide adaptors are ligated onto the end-repaired DNA fragment. These adaptors have a dual purpose. First, the adaptors allow for the attachment of the library to the MiSeq flow cell. The flow cell consists of a glass platform etched with microfluidic channels. Each flow cell contains covalently bound oligonucleotides that are complementary to the library adaptors. Second, the adaptors contain a sequencing primer annealing site. After adaptor ligation, barcodes are then ligated onto the fragmented amplicon. Each barcode consists of a unique six-base DNA sequence. The purpose of indexing is so that multiple patient samples can be combined for testing in a single NGS instrument run [5]. Successful end-repair, adaptor ligation, and final library concentration can be verified by qPCR using primers specific for the adaptors. Failure to amplify may indicate a low library concentration or may indicate that adaptors did not ligate properly.

Multiple steps are involved in HLA typing by NGS, and each laboratory is required to validate and optimize each one of these steps before this technique can be used for clinical purposes. In this study, we report results of the investigation of three important questions in the process of optimization of two-field HLA typing by NGS: the effects of fragment size for library preparation, indexing strategy, and read length on HLA typing.

2. Materials and methods

2.1. DNA samples and PCR amplification

Eight IHWG DNA samples (IHW09237, IHW09032, IHW09380, IHW09263, IHW09024, IHW09215, IHW09377, IHW09267) were used in this study. In-house designed primers were used for HLA-A (F 5′ ACT CTC TGG CAC CAA ACT CC, R 5′ TCT CAC CAG GGC TGC TTG), and previously published primers were used for HLA-B and C [12]. PCR conditions for A locus: Denature at 95° for 5 min followed by 35 cycles of the following: 94° for 30 s, 62° for 45 s, 72° for 5 min. An extension of 72° for 7 min follows. We amplified HLA-B and C as described [12]. HLA–DRB1 and DQB1 primers came from the commercial kit NGS-go (GenDx). Sizes of the PCR products ranged from 3900 bp to 4800 bp. Following PCR of IHWG DNA samples, DNA products were confirmed by gel electrophoresis and then purified (Qiaquick PCR Purification kit, Qiagen). Purified amplicons were then quantitated with the Nanodrop (Thermo Scientific). See Fig. 1 for procedure steps. Amplicons for individual indexing were adjusted to an input concentration of 0.75 μg. The five amplified loci from each sample for pooling were each adjusted to a concentration of 0.15 μg and pooled.

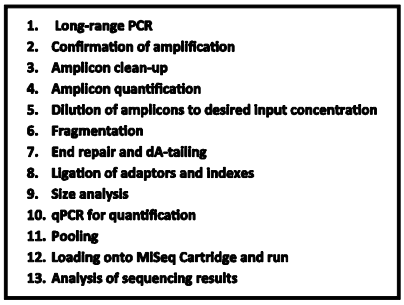

Fig. 1. Flowchart of library preparation for HLA NGS.

2.2. Library preparation and sequencing

The purified and diluted HLA amplicons were incubated with the NEB dsDNA Fragmentase Kit reagents following the manufacturer's directions (New England Biolabs). To generate two different fragment sizes, the PCR products were incubated for 30 or 20 min for 100–300 bp and 300–600 bp fragments, respectively. Library preparation steps of end-repair and adaptor ligation was completed using the NEBNext DNA Library Prep Master Mix Set for Illumina (New England Biolabs) following the manufacturer's directions. Indexes were added next using NEBNext Multiplex Oligos for Illumina (Dual Index Primer Set 1), (New England Biolabs) and two strategies were compared (see Fig. 2). Index-by-amplicon, where each individual fragmented amplicon is barcoded and index-by-patient, where each patient's fragmented five amplicons are equimolarly pooled after PCR and indexed with the same barcode. Following adaptor and index ligation, the libraries were evaluated for size (Caliper LabChip GX, PerkinElmer) and quantified by qPCR (Kapa Library Quantification Kits for Illumina). Manufacturer recommended steps to clean up library after each modification using AMPure XP Beads (Beckman Coulter) were followed. After quantification, each library was diluted and equimolarly pooled so that the total final concentration of input DNA was 2 nM. Manufacturer directions were followed in preparing the final library for addition to the MiSeq reagent cartridge. Final dilution of the library was 10 pM. Two MiSeq Reagent kits were evaluated: the v2 2 × 150 (300 cycle) and the v2 2 × 250 (500 cycle) kits (Illumina) in order to compare the effect of read length on HLA typing.

2.3. Sequence analysis

Sequencing results were analyzed using Omixon HLA Target Software (Omixon), database version 1.8.1. For sequence determination, we analyzed only the exons and followed the manufacturer's recommended settings of processing a maximum number of reads at 20,000 and 100,000 reads for the index-by-amplicon and index-by-patient groups respectively. Using the Omixon HLA Target Software, an unambiguous HLA typing result was defined when there was only one allele pair possible. Ambiguous typings were called when the correct alleles were present but other alleles could not be ruled out. Missed alleles were called when the software failed to identify the correct allele, and the HLA assignment was considered a fail when no HLA assignment could be made.

3. Results

3.1. PCR amplification and library preparation

Amplification of all samples was confirmed by gel electrophoresis (data not shown). Given that large whole-gene PCR amplicons must be first fragmented for Illumina sequencing [10], we used an endonuclease digestion approach to generate random overlap-

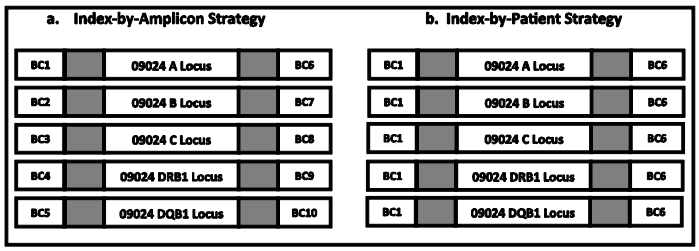

Fig. 2. Indexing strategy. Panel a is the configuration of the indexes used in our index-by-amplicon group. Each DNA fragment is individually tagged with a unique index (BC1–10) even though all amplicon fragments come from the same IHWG sample. Panel b is the configuration of indexes used in our index-by-patient group. Each DNA fragment from each IHWG sample is tagged with the same barcode.

Table 1
Results of fragmentation. Fragment sizes were determined by the Caliper LabChip GX (PerkinElmer). The average range of fragments is the smallest to largest size detected.

Desired fragment size/indexing strategy	Average size with adaptors	Range of fragments with adaptors	Average size without adaptors (140 bp)
100–300 bp, index-by-amplicon	341 bp	301–419 bp	201 bp
100–300 bp, index-by-patient	327 bp	301–347 bp	187 bp
300–600 bp, index-by-amplicon	692 bp	511–1061 bp	552 bp
300–600 bp, index-by-patient	702 bp	570–829 bp	562 bp

ping fragments of either 100–300 bp or 300–600 bp sizes in preparation for paired-end 150 bp and 250 bp read-length sequencing. The dsDNA Fragmentase Kit (New England Biolabs) uses two proprietary endonucleases consisting of *Vibrio vulnificus* nuclease, which randomly nicks one strand of DNA, and a modified T7 endonuclease, which cuts the opposite DNA strand, thus producing double-stranded DNA [13]. The fragment size resulting from endonuclease fragmentation was assessed prior to sequencing. The average size of the 100–300 bp fragment group was 339 bp, whereas the average size of the 300–600 bp fragment group was 694 bp (see Table 1). Library sizes represent the DNA fragment plus the 140 bp adaptor and index oligonucleotides. Subtracting the adaptors yields fragment sizes of 199 bp and 554 bp, respectively, which are within the target range. In conclusion, the New England Biolabs dsDNA Fragmentase Kit performed as expected.

DNA libraries were quantified after fragmentation and library preparation. The average DNA library concentration as determined by qPCR was 3.27 nM, with concentrations ranging from 0.05 nM to 18.06 nM. Two samples had low concentrations of 0.05 nM but we opted to add them to the final pooled library to investigate the lower limit of detection concentration. Both of these samples failed. The next lowest DNA library concentration was 0.27 nM and this sample typed correctly with adequate coverage. The kit performed as desired but overall, we found this method to be time- and labor-intensive requiring numerous steps to purify the DNA library.

3.2. Illumina MiSeq sequencing

Two MiSeq Reagent kits (2 × 150 and 2 × 250) were assessed in order to evaluate the effect of read length on HLA typing of the 96 pooled samples. Quality scores, coverage, and allelic balance were used to measure sequencing quality [14]. A Q30 score indicates that the nucleotide base has a 1 in 1000 probability of being called incorrectly and is similar to the Phred scores used in Sanger sequencing [15]. The Illumina MiSeq base calling quality score Q30 for the 2 × 250 and 2 × 150 runs were 85.7% and 93.1% respectively. Coverage refers to the number of times an individual base is sequenced and is essential for confidence of base call assignment. The overall coverage, as calculated by the Omixon software, of all samples was well above 100 (see Table 2). Under the conditions used, we could pool more samples and still achieve a satisfactory read depth. Finally, the allele pairs were balanced, each allele being individually represented between 30% and 70%. Theoretically, all alleles would be 50% but it is more typical for the allele balance to range between 30% and 70% for HLA. It has been suggested that NGS data accuracy decreases when allele balances range outside of the 20–80% range [16]. Most allele pairs in our study ranged between 40% and 60%. There were only two samples close to the limits: one allele pair had a 32%/68% allele balance in the index-by-amplicon group, and another allele pair had a 31%/69% allele balance in the index-by-patient group. We conclude

Table 2
Comparison of average overall coverage. Comparison of average overall coverage over all exons between different groups.

Group	A	B	C	DRB1	DQB1
Index-by-amplicon					
100–300 bp, 2 × 150	322 ± 63	486 ± 133	415 ± 172	423 ± 219	646 ± 186
300–600 bp, 2 × 150	412 ± 110	624 ± 177	678 ± 234	540 ± 141	752 ± 260
100–300 bp, 2 × 250	209 ± 42	304 ± 113	252 ± 108	214 ± 53	304 ± 69
300–600 bp, 2 × 250	561 ± 149	848 ± 231	978 ± 306	735 ± 225	995 ± 378
Index-by-patient					
100–300 bp, 2 × 150	240 ± 93	424 ± 99	475 ± 126	619 ± 341	914 ± 312
300–600 bp, 2 × 150	268 ± 141	398 ± 186	501 ± 264	465 ± 273	894 ± 443
100–300 bp, 2 × 250	137 ± 79	180 ± 103	241 ± 116	239 ± 158	367 ± 202
300–600 bp, 2 × 250	397 ± 209	556 ± 259	776 ± 395	682 ± 39	1179 ± 658

Table 3
Effect of fragment size on HLA typing. Comparison of fragment size groups only. An unambiguous HLA typing result was defined when there was only one possible allele pair without any ambiguous alleles that could not be ruled out. Ambiguous typing was called when the correct alleles were present but other alleles could not be ruled out. Missed alleles were called when the software failed to identify the correct allele, and the HLA assignment was considered as a fail when no HLA assignment could be made.

		A	B	C	DRB1	DQB1	Total (%)
(a) Index-by-amplicon group total HLA typing results							
100–300 bp groups	Unambiguous	32	24	29	29	28	142 (88.8%)
	Ambiguous	0	4	3	1	4	12 (7.5%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	4	0	2	0	6 (3.8%)
300–600 bp groups	Unambiguous	32	30	30	32	29	153 (95.6%)
	Ambiguous	0	2	2	0	3	7 (4.4%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)
(b) Index-by-patient group total results							
100–300 bp groups	Unambiguous	28	28	27	29	27	139 (86.9%)
	Ambiguous	3	4	5	3	5	20 (12.5%)
	Missed allele	1	0	0	0	0	1 (0.6%)
	Failed	0	0	0	0	0	0 (0%)
300–600 bp groups	Unambiguous	31	31	28	32	31	153 (95.6%)
	Ambiguous	1	1	4	0	1	7 (4.4%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)

that the quality metrics for our run based on Q30 scores, coverage, and allelic balance were all acceptable for testing.

3.3. HLA typing analysis

After sequencing, MiSeq fastq files were processed and analyzed using Omixon HLA Target Software (Omixon). To analyze, we opted to reduce the number of reads processed to 20,000 and 100,000 for the index-by-amplicon and index-by-patient groups, respectively. We found that 96% of all samples that were in the 300–600 bp size range could be HLA typed unambiguously. This is in contrast to the samples in the 100–300 bp range where only 88.8% and 86.9% of the indexed-by-amplicon and indexed-by-patient groups had an unambiguous typing, respectively (see Table 3). There were more ambiguous allele combinations present when the smaller fragments were sequenced. For example, in the 100–300 bp libraries, ambiguous alleles ranged from 6.3% to 17.5% whereas in the 300–600 bp libraries, the ambiguous allele rate was from 2.5% to 6.3% (Fig. 3 and Table 4). Overall, the group with the highest accuracy was the 300–600 bp, index-by-patient, 2 × 250 bp group with 97.5% unambiguous typing (Table 4). Six of the eight IHWG samples utilized here have previously been tested using NGS with the same results, thus confirming our typing assignments [6,7,25].

There was no difference in the 300–600 bp libraries for HLA typing in either the index-by-amplicon or index-by-patient groups suggesting that indexing-by-patient is a viable option (see Table 4). Surprisingly, there was no significant difference in HLA typing between the Illumina MiSeq v2 Reagents 2 × 150 versus 2 × 250 cycle kits, although, overall, the smaller libraries (100–300 bp) in combination with the larger read length kit had the most ambiguous typing (Table 4). This observation suggests that the fragments are not long enough to achieve phasing, resulting in more ambiguity. In conclusion, the biggest factor influencing HLA typing results in our experiment was that of fragment size.

4. Discussion

Sanger sequencing is the current gold standard for allele resolution HLA typing. HLA typing by NGS offers two main advantages over Sanger sequencing: (1) economical (low cost and high throughput), and (2) the clonal and quantitative nature of the result. In HLA typing by NGS, each library fragment is individually sequenced, providing data for each allele, therefore, the differences between each parentally inherited haplotype can be determined without ambiguity [5]. Currently, HLA typing results by Sanger sequencing are primarily based on sequence information of the HLA peptide-binding site, encoded by exons 2 and 3 in HLA class I and exon 2 in HLA class II genes. This may lead to mischaracterization of HLA alleles, especially, in minorities [17]. Recently, the National Marrow Donor Program undertook an initiative to evaluate five population-specific alleles that were often mistyped. They found varying degrees of discrepancies (13–86%) in the assignment of these alleles, meaning that patients needing these alleles face more obstacles finding a match [17]. The economic advantage of NGS over Sanger sequencing is that the entire gene can be sequenced, not just the peptide-binding site; therefore, more complete HLA allelic assignments can be made [18]. A recent publication suggests that the number of HLA alleles may be over 3.5 million per loci [19]. Moreover, alleles we currently think as the most common may indeed be not. For example, DRB1*14:54 is far more common than DRB1*14:01 [20] yet DRB1*14:01 is typically assigned because current typing methods only type exon 2 for DRB1. However, the single nucleotide change that defines these

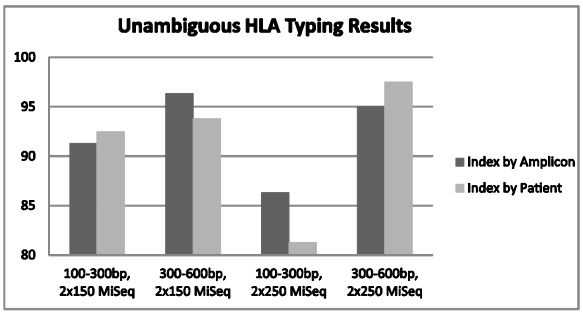

Fig. 3. Histogram of unambiguous HLA typing results. Comparison of the percentage unambiguous HLA typing between index-by-amplicon and index-by-patient groups. The four conditions are found in the legend of the *x*-axis. The 300–600 bp, 2 × 250, index-by-patient subset had the best overall unambiguous typing rate of 97.5%.

Table 4
HLA typing results. Results of indexed-by-amplicon (a) and indexed-by-patient (b) groups for both 2 × 150 and 2 × 250 v2 MiSeq Reagent kits. An unambiguous HLA typing result was defined when there was only one possible allele pair without any ambiguous alleles that could not be ruled out. Ambiguous typing was called when the correct alleles were present but other alleles could not be ruled out. Missed alleles were called when the software failed to identify the correct allele, and the HLA assignment was considered as a fail when no HLA assignment could be made.

		A	B	C	DRB1	DQB1	Total (%)
(a) Index-by-amplicon group HLA typing results							
100–300 bp, 2 × 150 MiSeq	Unambiguous	16	12	14	15	16	73 (91.3%)
	Ambiguous	0	2	2	1	0	5 (6.3%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	2	0	0	0	2 (2.5%)
300–600 bp, 2 × 150 MiSeq	Unambiguous	16	15	15	16	15	77 (96.3%)
	Ambiguous	0	1	1	0	1	3 (3.8%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)
100–300 bp, 2 × 250 MiSeq	Unambiguous	16	12	15	14	12	69 (86.3%)
	Ambiguous	0	2	1	0	4	7 (8.8%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	2	0	2	0	4 (5.0%)
300–600 bp, 2 × 250 MiSeq	Unambiguous	16	15	15	16	14	76 (95.0%)
	Ambiguous	0	1	1	0	2	4 (5.0%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)
(b) Index-by-patient group HLA typing results							
100–300 bp, 2 × 150 MiSeq	Unambiguous	15	14	14	16	15	74 (92.5%)
	Ambiguous	1	2	2	0	1	6 (7.5%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)
300–600 bp, 2 × 150 MiSeq	Unambiguous	15	15	13	16	16	75 (93.8%)
	Ambiguous	1	1	3	0	0	5 (6.3%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)
100–300 bp, 2 × 250 MiSeq	Unambiguous	13	14	13	13	12	65 (81.3%)
	Ambiguous	2	2	3	3	4	14 (17.5%)
	Missed allele	1	0	0	0	0	1 (1.3%)
	Failed	0	0	0	0	0	0 (0%)
300–600 bp, 2 × 250 MiSeq	Unambiguous	16	16	15	16	15	78 (97.5%)
	Ambiguous	0	0	1	0	1	2 (2.5%)
	Missed allele	0	0	0	0	0	0 (0%)
	Failed	0	0	0	0	0	0 (0%)

alleles lies in exon 3, which is outside the antigen recognition site and typically not sequenced. While this particular substitution may not have an effect on HSCT outcomes [20], other substitutions may. Generally, the clinical significance of alleles that only differ outside the peptide-binding site is unknown, and future studies that compare outcome data with complete HLA typing information obtained by NGS will answer this question. Finally, there are other benefits that may come with having a more complete reference HLA typing database, including a deeper understanding of population genetics, HLA expression, MHC evolution, and regulatory mechanisms [18]. Key to achieving this more complete and deeper understanding of HLA is that sequencing must be done on more than just a few exons. In our study, we performed long-range PCR of HLA-A, B, C, DRB1 and DQB1 loci. Due to the large size of intron 1, only exon 2 to the 3′UTR of DRB1 was amplified, whereas all the others were amplified from the 5′UTR to 3′UTR.

We selected the Illumina MiSeq as our sequencing platform because of its low error rate in comparison to other instruments [21]. The MiSeq works by a process known as sequencing by synthesis and there are numerous technical papers discussing the method [22,23]. To take advantage of the high sequencing capacity of the Illumina MiSeq, samples must be pooled. To determine the most ideal pooling strategy, we compared two different indexing strategies and their effect on HLA typing results. Indexing entails the ligation of a unique oligonucleotide, (often referred to as a barcode) on both ends of the adaptor-ligated DNA fragment. The HLA region is well-known for the homology that exists between each class I locus and between each class II locus. Evidence suggests that the different loci arose through gene duplication events [24], which could potentially hinder bioinformatics analysis of the sequencing results. For this reason, we compared ligating a unique index to each amplicon (index-by-amplicon group) versus ligating the same index to each of the five loci per patient sample (index-by-patient group). The HLA typing results obtained in our study as well as the quality of sequencing data confirmed that indexing-by-patient is a viable option, a conclusion that has also been reached by others in the field [25]. Indexing-by-patient results in considerable labor and cost-savings during the library preparation stage. Instead of having to handle each amplicon uniquely, the five amplicons from one patient can be pooled and handled as if it were one sample.

The large whole-gene PCR amplicons must be fragmented for Illumina sequencing, as Illumina MiSeq instruments can only generate short sequence reads [10]. The purpose of fragmenting the DNA is to generate overlapping sequences that can be tiled so that the entire amplified DNA sequence can be determined. Fragmentation can be done by several different methods including sonication, nebulization, or by enzymatic methods [26]. Within enzymatic fragmentation methods, several different classes of enzymes are available, including transposons [27] and endonucleases [28]. Sonication is often used and efficient in generating random fragments of the desired sizes, but currently requires manual processing and

cannot take advantage of the commonly used liquid handling laboratory automation, leading us to try enzymatic methods. We used an endonuclease digestion approach to generate random overlapping fragments of either 100–300 bp or 300–600 bp sizes for paired-end 150 bp and 250 bp read-length MiSeq sequencing. We found that endonucleases worked efficiently to fragment the long-range PCR amplicons into sizes between 100 bp and 600 bp. We have evaluated the effect of fragment sizes on HLA typing and found that 96% of all samples in the 300–600 bp fragment size range could be unambiguously typed using 250 bp-long paired-end sequencing. In contrast, there was an about 5% decrease in unambiguous typing of samples when the PCR amplicons were fragmented into the 100–300 bp range suggesting that larger fragments are better at resolving phasing ambiguities.

Our analysis software provided several options for performing HLA typing. In addition to the option of analyzing only the exons, other settings included options for analyzing exons, introns, and UTRs as well as the whole gene. We chose to analyze only the exons due to the paucity of intronic sequences in the IMGT database. This however, will change as the IMGT database becomes more complete in the future. We also chose to process more reads for the index-by-patient group than we did for the index-by-amplicon group because each patient sample was a pool of five loci (HLA-A, B, C, DRB1 and DQB1). This would ensure that adequate coverage was seen across the pool of all five loci.

In conclusion, we found our method of long-range PCR amplification of whole genes combined with enzymatic fragmentation to be a suitable method for HLA typing by Illumina NGS library sequencing. The presented approach resulted in typing of 96% of samples at the allele-level resolution required for HSCT without additional reflexive testing. We obtained the most correct and least ambiguous results when we used longer-sized fragments with longer read length sequencing kits. Additionally, we found that indexing-by-patient is a cost-savings technique for multiplexing numerous samples together without sacrificing typing quality. Downsides of our method include its time- and labor-intensiveness, but these drawbacks can be negated by the adoption of liquid handling automation. Overall, we find that HLA allele assignment by NGS provides more accurate, less ambiguous typing without the need for reflexive testing required with Sanger sequencing. We expect that performing this methodology will lead to minimizing expense and time delays associated with selecting the most optimal donor.

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CHAPTER 3

HLA GENOTYPING IN THE CLINICAL LABORATORY: COMPARISON OF NEXT-GENERATION SEQUENCING METHODS

HLA
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HLA genotyping in the clinical laboratory: comparison of next-generation sequencing methods

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Abstract

Implementation of human leukocyte antigen (HLA) genotyping by next-generation sequencing (NGS) in the clinical lab brings new challenges to the laboratories performing this testing. With the advent of commercially available HLA-NGS typing kits, labs must make numerous decisions concerning capital equipment and address labor considerations. Therefore, careful and unbiased evaluation of available methods is imperative. In this report, we compared our in-house developed HLA NGS typing with two commercially available kits from Illumina and Omixon using 10 International Histocompatibility Working Group (IHWG) and 36 clinical samples. Although all three methods employ long range polymerase chain reaction (PCR) and have been developed on the Illumina MiSeq platform, the methodologies for library preparation show significant variations. There was 100% typing concordance between all three methods at the first field when a HLA type could be assigned. Overall, HLA typing by NGS using in-house or commercially available methods is now feasible in clinical laboratories. However, technical variables such as hands-on time and indexing strategies are sufficiently different among these approaches to impact the workflow of the clinical laboratory.

Introduction

Human leukocyte antigen (HLA) genotyping by next-generation sequencing (NGS) has matured from proof-of-principle concept to the availability of several commercial typing kits all within the space of a few years (1–10). The greatest benefit of this new technology is the achievement of greater allele resolution with less ambiguity at a cost that is comparable, or even cheaper than current methods such as Sanger sequencing or Sequence-Specific Oligonucleotides (SSO)/Sequence-Specific Primers (SSP) (11). Single-molecule sequencing of the HLA genes will likely become the gold standard for HLA typing because all exons can be evaluated, not just a few, as done by Sanger sequencing. In addition, complete allele resolution will become possible as all exonic and intronic regions become fully characterized in the IPD-IMGT/HLA Database (12). The ambiguities found with current methods are primarily due to the inability of current technologies to provide phasing, the lack of reference sequences (13) and interrogation of only a selected few exons. Furthermore, this new technology has the potential to help improve the understanding of the biology of HLA that moves far beyond simple allele characterization and into the regulation, transcription, and expression of these genes (14). In the meantime, the challenge is the clinical implementation of the best-fit and most accurate method. The aim of this study was to evaluate an in-house developed method along with two recently available commercial kits for HLA typing by NGS on the Illumina MiSeq and compare the library preparation process, and concordance between final genotyping outcomes.

Material and methods

Samples

A total of 48 samples were compared by three different HLA genotyping methods for NGS. Of these 10 samples came from the International Histocompatibility Working Group (IHWG): (IHW09024, IHW09032, IHW09215, IHW09237, IHW09263, IHW09267, IHW09376, IHW09377, IHW09380, and IHW09385). The remaining 38 samples were de-identified DNA samples from the Histocompatibility and Immunogenetics laboratory at the University of Utah, randomly collected from potential hematopoietic stem cell transplant

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recipients and donors. DNA was extracted from whole blood using the automated Maxwell 16 Blood DNA Purification kit and the Maxwell 16 SEV instrument (Promega, Madison, WI). Initial HLA typing of the samples was performed by a combination of Sanger sequencing (Histogenetics LLC, Ossining, NY) and LABType SSO techniques (One Lambda, Canoga Park, CA).

In-house HLA NGS: targeted library size comparison

Our method for HLA genotyping by NGS has recently been reported (15). For the purpose of this study, we evaluated larger fragment sizes using the 10 IHWG samples listed above. Long-range PCR was performed as reported (15) for HLA-A, B, C, DRB1, and DQB1. Following PCR clean-up (QIAquick PCR Purification, Qiagen, Germantown, MD), each amplicon was individually quantified (Nanodrop) and each sample's five HLA genes (A, B, C, DQB1, DRB1) were pooled for a total DNA concentration of 0.75 µg. Enzymatic shearing of the DNA was performed using NEBNext dsDNA Fragmentase (New England BioLabs, Ipswich, MA). Incubation times were adjusted to target desired ranges (Table 1). Following fragmentation, end-repair, dA-tailing, and adaptor ligation were performed as directed using NEBNext DNA Library Prep Master Mix Set for Illumina (New England BioLabs). Three different size selection strategies were compared: AMPure Beads (Beckman Coulter, Brea, CA), Blue PippinPrep (Sage Science, Beverly, MA), and no size selection. Unique paired-end indices [NEBNext Multiplex Oligos for Illumina (Dual Index Primer Set 1), New England BioLabs] were ligated onto each sample and an eight cycle PCR enrichment step was performed. Each library was measured for size (Caliper LabChip GX, PerkinElmer, Waltham, MA) and quantified (Kapa SYBR FAST LightCycler 480 qPCR kit, KAPA Biosystems, Wilmington, MA). All samples were equimolarly pooled and two Illumina (San Diego, CA) MiSeq Reagent kits were run, v2 (2×250) and v3 (2×300), each loaded at 10 pM concentration. Fastq files were analyzed using Omixon's (Budapest, Hungary) Target v.1.8.1 software for HLA genotyping. Finally, six raw fastq files were randomly selected and trimmed to 100,000, 50,000, 10,000, 5000, 4000, 3000, 2000, and 1000 reads using the free online bioinformatic tool, 'seqtk', (http://ged.msu.edu/angus/tutorials-2013/seqtk_tools.html#id1). Each down-sampling of paired end reads was performed in triplicate using a different random seed for each replicate. These files were then processed by the Omixon Target v1.8.1 software to determine how many reads were needed for accurate genotyping.

In-house HLA NGS: comparison with commercial kits

Based on the targeted amplicon comparison of the 10 IHWG samples, the remaining 38 clinical samples were run using the 600–900 bp, PippinPrep size selection, 2×250 MiSeq read-length kit conditions. Two samples of the 38 clinical samples had insufficient quantity for evaluation with the in-house method. All 36 samples were loaded onto the MiSeq reagent cartridge.

Table 1 Targeted library size groups with incubation times and size selection method

Targeted size group (bp)	Fragmentase incubation time (min)	Size selection method
600–900	15	No size selection
600–900	15	PippinPrep
600–900	15	AMPure Bead
600–1200	9	PippinPrep
900–1200	5	PippinPrep

bp, base pair.

Omixon Holotype ×2 24/7

The Omixon Holotype ×2 24/7 kit was used according to the manufacturer's instructions. This kit provides typing reagents for HLA-A, B, C, DQA1, DQB1, and DRB1 genotyping; however, for the purpose of this evaluation, only HLA-A, B, C, DRB1, and DQB1 genotyping results were assessed. Following PCR amplification, library preparation, sample indexing, and size selection, samples were pooled and loaded onto the Illumina MiSeq using the 2×250 read-length kit. A total of 24 samples were loaded at a time on a MiSeq reagent cartridge. After sequencing, fastq files were analyzed by kit-supplied HLA Twin software program, v1.1.

Illumina HLA TruSight v1

Manufacturer's instructions were followed for the performing HLA-A, B, C, DPA1, DPB1, DQA1, DQB1, and DRB typing using the Illumina HLA TruSight v1 kit; however, only HLA-A, B, C, DQB1, and DRB1 typing results were used for the purpose of kit comparison. Following PCR amplification, fragmentation of the long-range PCR product occurs by transposons (referred to as 'Tagmentation' by the manufacturer) in which the amplicon is fragmented, end-repaired, and adaptor ligated in one step. Next, libraries are purified and indexed in preparation for sequencing. The last step consists of a clean-up step and library normalization. The bead normalization simultaneously performs an amplicon clean-up as well as size selection. Samples were pooled and then loaded onto the Illumina MiSeq using the 2×250 read-length kit. A total of 24 samples were loaded at a time on a MiSeq reagent cartridge. Fastq files were analyzed using the kit-supplied CONEXIO ASSIGN software program, v1.0.0.729.

Analysis and method comparison

Results for all three methods were evaluated for overall accuracy, ambiguity at the second field, metric failures due to low

Table 2 Comparison of targeted library sizes[a]

Targeted size group (bp)	Size selection method	Average fragment size (bp)	Fragment size range (bp)	Average qPCR Conc. (nM)
600–900	No size selection	417	366–469	91.8±23.4
600–900	PippinPrep	627	596–666	50.0±20.1
600–1200	PippinPrep	677	614–779	43.2±15.8
600–900	AMPure Bead	789	672–977	12.2±4.6
900–1200	PippinPrep	973	650–1147	4.7±1.6

bp, base pair.

[a]The average fragment sizes from each target group are listed, measured by the Caliper LabChip GX (PerkinElmer). Fragment sizes include the Illumina adaptor and dual indexes (140 bp). Average concentration and one standard deviation of library is shown as measured by qPCR.

Q30 scores or low coverage, failure to genotype and finally, problems in software calling of alleles. Both the Omixon Holotype ×2 24/7 and Illumina HLA TruSight v1 kits came with proprietary software which were used as indicated. The in-house method was analyzed using the standalone software from Omixon, Target v1.8.1. In addition, each library preparation method was compared for ease of use and ramifications for clinical lab implementation.

Results

In-house HLA NGS: targeted library size comparison

We have recently reported the details of an in-house developed HLA NGS genotyping method (14). In this prior work, fragment sizes of 100–300 bp and 300–600 bp were compared using two different MiSeq Reagent (Illumina) read-length kits (2×150 and 2×250). In the current study, we further optimized fragment size and read-length to decrease ambiguous results because of insufficient phasing associated with shorter DNA fragments. A total of 10 IHWG samples were used for read-length optimization. The in-house method uses long-range PCR to amplify the entire HLA-A, B and C gene, and exons two through four for DRB1 and DQB1. Amplification was confirmed by gel electrophoresis (data not shown). Because each amplicon ranged in size from 3900 to 4800 bp, each PCR product must be fragmented to be suitable for the Illumina sequencers (16). Using partial endonuclease digestion, the fragment size is dependent on the length of incubation time of the PCR product with the enzymes. The Fragmentase kit makes use of two proprietary endonucleases, one of which will randomly nick the double-stranded DNA and the other will cut the opposing strand in the same location (17). Determining the ideal fragment size is an important consideration for HLA genotyping by NGS because longer sequences are more able to span distant polymorphisms in phase and result in less ambiguity (18). The NEB protocol does not recommend AMPure bead size selection of DNA insert sizes greater than 450 bp (19, 20) so the PippinPrep was used to obtain fragments greater than 700 bp. The size of each library is presented in Table 2. We found little difference in average fragment sizes within the 600–900 bp (AMPure and PippinPrep size selection) and 600–1200 bp groups despite different incubation times with the Fragmentase (Table 2). There was also little difference in the unambiguous typing results between these three groups which suggests that either AMPure bead or PippinPrep size selection is suitable (Table 3). The only difference we found was that there was a greater loss of DNA in AMPure size-selected group compared with the PippinPrep group, as measured by qPCR (Table 2) although this loss did not directly affect accuracy of results in this small sample study. We opted to use the PippinPrep for our 36 clinical samples because of its load-and-walkaway feature although the AMPure Bead method could be automated. The smallest-size library, the 600–900 bp without size selection (no size select group in Table 3), had the most 2-field ambiguity of the five groups compared.

Another difficulty in obtaining larger fragments may occur during the enrichment PCR step. Because the starting DNA concentration is low (0.75 μg), we hypothesize that the PCR enrichment step used for index ligation is preferentially amplifying the smaller fragments. The No-Size-Select group supports this argument because this group had the same Fragmentase incubation time as the 600–900 bp groups yet the average fragment size was only 417 bp (Table 2). In this situation, the smaller fragments are not removed and thus are amplified at the expense of the much larger sized fragments. Future work will involve investigating the effect of increasing the starting DNA concentration of each amplicon to avoid smaller fragment bias as well as to eliminate or reduce the number of PCR cycles needed. In addition, PCR enrichment has the potential to introduce incorrect nucleotides into the DNA sequence. This could potentially have significant impact on the sequencing of the highly polymorphic HLA genes. Moreover, sequences that are GC rich may lead to allelic imbalance as the result of differential PCR amplification (16, 21). Of the three methods evaluated, only the Omixon Holotype ×2 24/7 kit does not require PCR for index ligation and library enrichment. The 900–1200 bp PippinPrep group produced the lowest library concentration indicating that either the Fragmentase incubation time or the size selection methods are not completely optimized for this fragment range.

Library quantification was determined by qPCR. Because the qPCR primers are homologous to the Illumina adaptors, amplification only occurs if adaptors were successfully

Table 3 Results of in-house HLA NGS: targeted library comparison[a]

	2×250 read-length group						2×300 read-length group					
	600–900 bp no size select						600–900 bp no size select					
	A	B	C	DRB1	DQB1	Total (%)	A	B	C	DRB1	DQB1	Total (%)
Unambiguous	16	18	18	20	19	91%	19	19	19	20	18	95%
Ambiguous	3	2	2	0	1	8%	1	1	1	0	1	4%
Incorrect allele	1	0	0	0	0	1%	0	0	0	0	1	1%
Failed	0	0	0	0	0	0%	0	0	0	0	0	0%
	600–900 bp AMPure bead size select						600–900 bp AMPure bead size select					
	A	B	C	DRB1	DQB1	Total (%)	A	B	C	DRB1	DQB1	Total (%)
Unambiguous	18	19	19	20	20	96%	18	20	19	20	20	97%
Ambiguous	1	1	1	0	0	3%	1	0	1	0	0	2%
Incorrect allele	0	0	0	0	0	0%	1	0	0	0	0	1%
Failed	1	0	0	0	0	1%	0	0	0	0	0	0%
	600–900 bp PippinPrep size select						600–900 bp PippinPrep size select					
	A	B	C	DRB1	DQB1	Total (%)	A	B	C	DRB1	DQB1	Total (%)
Unambiguous	20	20	18	20	20	98%	19	20	19	20	20	98%
Ambiguous	0	0	2	0	0	2%	1	0	1	0	0	2%
Incorrect allele	0	0	0	0	0	0%	0	0	0	0	0	0%
Failed	0	0	0	0	0	0%	0	0	0	0	0	0%
	600–1200 bp PippinPrep size select						600–1200 bp PippinPrep size select					
	A	B	C	DRB1	DQB1	Total (%)	A	B	C	DRB1	DQB1	Total (%)
Unambiguous	18	20	19	20	20	97%	18	20	18	20	20	96%
Ambiguous	0	0	1	0	0	1%	0	0	2	0	0	2%
Incorrect allele	0	0	0	0	0	0%	0	0	0	0	0	0%
Failed	2	0	0	0	0	2%	2	0	0	0	0	2%
	900–1200 bp PippinPrep size select						900–1200 bp PippinPrep size select					
	A	B	C	DRB1	DQB1	Total (%)	A	B	C	DRB1	DQB1	Total (%)
Unambiguous	17	20	19	20	20	96%	17	20	18	20	20	95%
Ambiguous	1	0	1	0	0	2%	1	0	2	0	0	3%
Incorrect allele	0	0	0	0	0	0%	0	0	0	0	0	0%
Failed	2	0	0	0	0	2%	2	0	0	0	0	2%

bp, base pair; HLA NGS, human leukocyte antigen-next generation sequencing; IHWG, International Histocompatibility Working Group.
[a]Results of 2×250 and 2×300 MiSeq read-length kits. 'Unambiguous (2 field)' indicates the number of alleles that genotyped without any ambiguity at the 2-field level. 'Ambiguous (2 field)' is the number of alleles that genotyped with an ambiguity at the 2-field level. 'Missed Allele Call' is defined as the number of alleles that typed incorrectly. 'Failed' indicates the number of alleles that did not genotype. The dataset represents 10 IHWG samples, or 20 alleles per locus.

ligated onto the DNA fragment and thus, it serves as a good indicator of library preparation robustness. The average library concentration was 42.7 nM with concentrations ranging from 2.8 to 148 nM. The 600–900 bp no size select group had the largest concentration of DNA, and the 900–1200 bp PippinPrep group had the lowest overall DNA concentration of 4.7 ± 1.6 nM, suggesting that DNA loss occurs during the size selection process.

Overall, most of the groups offered a very high percentage of correct, unambiguous genotyping for both the 2×250 and 2×300 read length kits. The No-Size-Select group in the 2×250 read length kit performed the poorest with only 91% of all alleles typing correctly and unambiguously (Table 3). This group also had the greatest number of ambiguous allele combinations and the lowest overall-fragment size which suggests that the smaller fragments prevented phasing. The 600–900 bp, PippinPrep size selected, 2×250 read length group performed the best with 98% of all alleles typing correctly and unambiguously. Surprisingly, there was no difference in this group between the 2×250 and 2×300 read-length kits. In theory, there should be less ambiguity with a longer read-length; however, this was not observed in our limited data set. Therefore, we chose to use the 2×250 read length kit because it has a much shorter run time on the MiSeq, 40 h as opposed to 60 h required by the 2×300 read length kit. Based on the results of genotyping, as well as time and labor considerations, we genotyped the 36 clinical samples using the 600–900 bp, PippinPrep size selection, and the 2×250 read length kit.

Comparison of the in-house method with commercial kits

A total of 36 clinical and 10 IHWG samples were genotyped by three different HLA NGS typing methods: Omixon Holotype ×2 24/7 kit, Illumina TruSight v1 HLA Sequencing kit, and the in-house developed HLA typing method primarily using reagents from New England BioLabs.

Genotyping data for HLA-A, B, C, DRB1, and DQB1 were compared for this study (Table 4). Overall, 97.2% of alleles genotyped by the in-house method were unambiguous and correct at the second field, followed by Omixon Holotype with 95.6% and lastly, the Illumina HLA TruSight v1 kit with 82.5%. The appeal of using single molecule sequencing for HLA is its potential to resolve ambiguity, which has been the most vexing problem of the HLA field. The HLA Illumina TruSight v1 kit had the highest number of ambiguous allele combinations. Over 6% of all alleles had ambiguity at two-field, with the DRB1 gene showing the highest number of ambiguous allele combinations at 26% of all DRB1 alleles typed. Fortunately, most of the ambiguities involved alleles that are not common and well-documented (CWD) and thus would not require resolution (22). There were also more mismatches or phasing issues with the Illumina Conexio Assign software, although, improvements in their software are in development (Illumina, personal communication). Nearly 96% of all samples typed correctly and unambiguously with the Omixon Holotype kit.

Evaluation of 36 clinical samples using the in-house HLA NGS method

Overall, our in-house method performed well, however, there were some problems with resolving the alleles *C*04:01:01* and *C*04:09N* using the Omixon Target software, although the Omixon Twin software was able to rule out the presence of *C*04:09N* in the same samples. This particular ambiguity is undesirable because the NMDP policy for confirmatory typing requirements specifically requires resolution of *C*04:09N* when it exists as a possibility within a G group (23). The primary concern with the in-house method is that it is labor intensive, requiring numerous manual clean-up steps (Table 5). Although the index-by-sample strategy reduces the number of libraries to prepare, the number of clean-up steps creates a significant number of extra pipetting steps. Further optimization could involve reducing the number of clean-up steps, and employing automation.

Evaluation of Illumina HLA TruSight v1

The Illumina HLA TruSight v1 kit uses long-range PCR to generate whole-gene amplicons for HLA-A, C, DQA1 and DPA1, and most exons for B, DRB1/3/4/5, DQB1, and DPB1. Fragmentation makes use of transposons to simultaneously fragment, end-repair, dA-tail, and add adaptors to the amplicons. This is in contrast to other fragmentation methods such as sonication and enzymatic steps which do require separate steps for end-repair, dA-tailing, and adaptor ligation. The time saved by the use of transposons is lost due to the index-by-amplicon approach in which each amplicon must be individually handled throughout the entire procedure until the last step before loading of the MiSeq cartridge (Table 5). This strategy required a significant amount of pipetting. In fact, the Illumina v1 kit used the greatest number of tips, 3146 for the five HLA genes compared with Omixon (587 tips) and in-house (1901 tips). Both Omixon and the in-house methods use an index-by-sample strategy wherein, after post-PCR quantification, the amplicons for each gene for each sample are pooled. This strategy significantly reduces the amount of pipetting required.

The DRB1 gene had the highest number of ambiguous allele combinations, although, nearly all of the ambiguities involved alleles not on the common and well-documented CWD allele list (22). Many of the DRB1 ambiguities occurred for alleles with a single base pair change at the start of exon 2. This is probably a primer issue for DRB1, which could be solved by the manufacturer by re-positioning this primer. Illumina is in process of developing a version 2 kit which will address many of these issues including changing their indexing strategy to an index-by sample approach (Illumina, personal communication).

Omixon Holotype ×2 24/7

Similar to the other methods, the Omixon Holotype ×2 24/7 kit employs long-range PCR to amplify the entire gene for HLA-A, B, C, DQB1, DQA1, and partial amplification for DRB1 and DPB1. After amplification, PCR products are measured using a dye that is specific for double-stranded DNA and then each sample's seven loci are equimolarly pooled together. This index-by-sample approach greatly reduces the amount of pipetting. For example, 24 samples with 7 loci apiece, or 168 wells, becomes just 24 wells to complete library preparation. By employing this strategy, the overall cost of the assay is reduced because less consumables and labor are required yet robust and accurate HLA genotyping is still achieved.

Concordance between the three methods

Overall, there was good concordance between the three methods but a few issues such as metric failures (low Q30 or low coverage) or failure to type precluded complete concordance. In addition, the Illumina Assign software had a number of allele assignments where base pair or phasing mismatches prevented full allele assignment (5% of all alleles). In a few cases there was some ambiguity present but this was generally due to suspected primer placement. The Illumina kit had more two-field ambiguity than the other two methods, especially for B and DRB1 genes (6.3%). The Omixon Holotype kit did not have any two field ambiguity but there was some ambiguity in the fourth field which represents intronic variants. One of the clinical samples was reported to have a novel C locus allele by the

Table 4 Comparison of three methods[a]

Illumina HLA TruSight	A		B		C		DQB1		DRB1		Total	
48 samples	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Unambiguous at second field	86	89.6	79	82.3	88	91.7	78	81.3	65	67.7	396	82.5
Ambiguous at second field	0	0.0	3	3.1	0	0.0	2	2.1	25	26.0	30	6.3
Incorrect	0	0.0	0	0.0	0	0.0	0	0.0	2	2.1	2	0.4
Metric failure	2	2.1	0	0.0	0	0.0	4	4.2	0	0.0	6	1.3
Failure (no results)	2	2.1	2	2.1	2	2.1	12	12.5	4	4.2	22	4.6
Mismatch or phasing issue	6	6.3	12	12.5	6	6.3	0	0.0	0	0.0	24	5.0
Omixon Holotype x2 24/7	**A**		**B**		**C**		**DQB1**		**DRB1**		**Total**	
48 samples	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Unambiguous at second field	96	100.0	96	100.0	95	100.0	94	97.9	78	81.3	459	95.6
Ambiguous at second field	0	0.0	0	0.0	0	0.0	2	2.1	0	0.0	2	0.4
Incorrect	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Metric failure	0	0.0	0	0.0	0	0.0	0	0.0	10	10.4	10	2.1
Failure (no results)	0	0.0	0	0.0	0	0.0	0	0.0	8	8.3	8	1.7
Mismatch or phasing issue	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	1	0.2
In-House, 600–900 bp, PippinPrep, 2x250	**A**		**B**		**C**		**DQB1**		**DRB1**		**Total**	
46 samples	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Unambiguous at second field	92	100.0	92	100.0	87	94.6	90	97.8	86	93.5	447	97.6
Ambiguous at second field	0	0.0	0	0.0	4	4.3	0	0.0	0	0.0	4	0.9
Incorrect	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Metric failure	0	0.0	0	0.0	0	0.0	0	0.0	2	2.2	2	0.4
Failure (no results)	0	0.0	0	0.0	0	0.0	2	2.2	4	4.3	6	1.3
Mismatch or phasing issue	0	0.0	0	0.0	1	1.1	0	0.0	0	0.0	1	0.2

bp, base pair.

[a]Results of three methods compared. Each sample was evaluated for overall accuracy, ambiguity (resolution at 2-field allele level), metric failures (low sample coverage, low Q30, or other flag indicated in software), failure to type, or mismatch or phasing issue with allele sequence.

Omixon Twin software while the Illumina Conexio Assign software reported it having a one bp mismatch with the reference sequence in exon 4. The Omixon Target software did not flag this sample in any way as being novel or having a mismatch and it was only after seeing the Twin and Assign results, that re-inspection of the actual sequence in the Target software confirmed the base pair substitution.

Comparison of resolution was limited to the second field due to the inability of the in-house-used Omixon Target software to make third field calls for some alleles. Future work will entail comparison at a higher resolution level. With the exception of the IHWG samples, the samples used in this study are representative of the HLA genotypes commonly seen by our bone marrow transplantation program, and did not contain any particularly unusual HLA types.

Genotyping results of the IHWG samples

The IHWG samples genotyped correctly, although there were two discrepancies with the reported results (Table S1, Supporting Information). DRB1 typing for IWH09377 (FH05) is reported as '*DRB1*14:01:01*' but all three methods genotyped this allele as *DRB1*14:54:01*. Only one base pair differentiates *DRB1*14:01:01* from *DRB1*14:54:01* and this difference is in exon 3, which is traditionally not sequenced. Other reports have confirmed this finding (24). Another discrepancy was found in IHW093858 (FH11). IHWG reports this as *DRB1*11:01:02* but our three methods had complete concordance for *DRB1*11:01:01*, which has been confirmed by NGS by two other labs (3, 25). We were able to confirm genotyping for IHWG09237 (26), IHWG09263, IHWG9024 (24), IHWG09032 and IHWG09215 (3) and they were consistent with previous reports.

Sequence analysis and software

Coverage is defined as the number of times an individual base is sequenced and the three software packages evaluated in our study report the overall average depth. Overall read depth is shown in Table 6 with the in-house method resulting in the highest read depth per base, while the Omixon Holotype method resulted in the lowest. Establishing adequate coverage depth is important for assuring confidence in sequencing base calls, and maximizing the number of individual samples sequenced in one run. Unfortunately, increasing either sample number or coverage comes at the expense of the other (27). This is of concern because the overall cost per sample decreases when the number of samples per run is increased. Currently, a 10× to

Table 5 Method comparison of library preparation steps

Step	Omixon Holotype x2 24/7	In-house 24 samples	Illumina HLA TruSight v1 24 samples
Post PCR clean-up	Not required	QIAquick PCR Purification	Magnetic beads (manual)
Amplicon quantification	Promega Quantifluor Dye with standards on fluorometer	Nanodrop or Qubit	Bead normalization. No measurement of amplicons required
Post-pooling clean-up	Pool amplicons and ExoSAP-IT (hands off incubation)	Pool amplicons only. No clean-up	Done in above step
Fragmentation	25-min incubation in thermocycler	15-min incubation in thermocycler	12-min incubation in thermocycler
Post-fragmentation cleanup	Not required	QIAquick PCR purification	Magnetic beads (manual)
End-repair	1-h incubation in thermocycler	30-min incubation in thermocycler	Not required
Clean-up step	Not required	Magnetic beads (manual)	Not required
dA-Tailing	Not required	30-min incubation in thermocycler	Not required
Clean-up step	Not required	Magnetic beads (manual)	Not required
Index adaptor ligation	30-min incubation in thermocycler	30-min incubation in thermocycler	12 PCR cycles
Pool samples and clean-up	Pool samples and magnetic beads (manual)	Magnetic beads (manual). Samples not pooled at this step	Magnetic beads (manual)
Size selection	PippinPrep size select 650–1300 bp	PippinPrep size select 600–900 bp	Bead size selection and normalization done in above step
PCR enrichment	Not required	Eight PCR cycles	Not required
Post enrichment clean-up	Not required	Magnetic beads (manual)	Not required
qPCR	Kapa Library Quantification	Kapa Library Quantification	Not required
Sample pooling	Previously done	All samples pooled	Pool all amplicons and samples and run on Qubit
MiSeq	2x250 (40-h run)	2x250 (40-h run)	2x250 (40-h run)

HLA, human leukocyte antigen.

Table 6 Coverage comparison[a]

	Coverage		
	In-house A	Omixon A	Illumina A
Average	585.2	99.3	305.2
SD	390.8	36.9	22.7
	B	B	B
Average	842.8	94.5	309.1
SD	325.1	29.5	28.6
	C	C	C
Average	941.3	104.1	308.6
SD	374.3	30.9	34.4
	DQB1	DQB1	DQB1
Average	970.2	121.8	268.7
SD	636.5	41.9	37.2
	DRB1	DRB1	DRB1
Average	697.6	70.0	285.0
SD	613.7	32.1	90.3

[a]Summary of the average and standard deviations of the overall coverage found in each sample.

30× depth of coverage is required for human genome variants or single-nucleotide polymorphisms (28). The Omixon Twin software flags any sample with coverage less than 25 copies per base and suggests an 'investigation' of samples with coverage of 10–25. Samples are flagged as a fail if coverage is less than 10. The Omixon Target software, which was used for the in-house analysis reports out alleles with coverage greater than 10 without any flags, whereas the Illumina Conexio Assign software flags any sample with coverage less than 50. Given this ambiguity in recommended minimal read depth, we became interested in the lower limit of required reads for accurate typing using the in-house method.

For the in house run, the average number of reads used for input into the analysis across the 36 samples was 392,562. To estimate the lower limit for the number of reads required for accurate typing, we performed a series of *in silico* down-sampling experiments on six of the samples. In this experiment, the fastq files were randomly trimmed to 100,000, 50,000, 10,000, 5,000, 4,000, 3000, 2000, and 1000 reads in order to approximate increased multiplexing and lower per sample coverage. Each down-sampling was performed in triplicate with replicates containing the same number of reads but a different random sampling of the full read dataset. The data was analyzed using the Omixon Target software. We found that in five out of six samples, 100% of the typings were correct and unambiguous when downsampling was limited to 4000 reads or more (Figure 1). In this experiment, we found that the software operated mostly in a binary fashion: when less than 4000 reads were processed, alleles were either reported correctly or were not reported at all. Theoretically, in heterozygotes, each allele is represented by 50% of the sequences in each sample, but we found that most of the heterozygous alleles in this study ranged

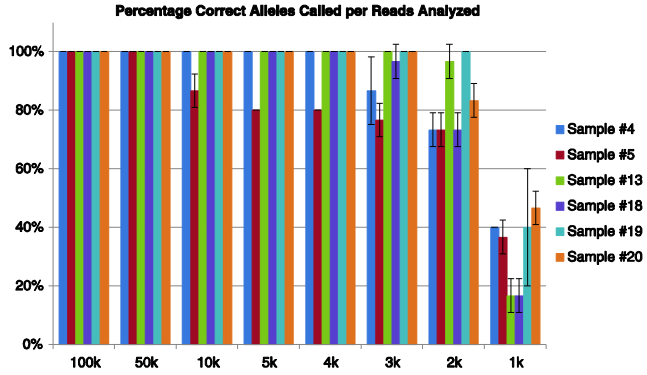

Figure 1 Percentage of correct alleles assigned per number of reads analyzed. Bar graph showing decrease in percentage of alleles called per number of reads processed. Reads were trimmed and then analyzed on the Omixon Target software. No incorrect alleles were assigned by the software although sample #5 had some allele dropouts at the lower number of reads. Error bars show 1 SD across the three seeds per read analyzed.

from 30 to 70%. There was one sample with a DQB1 allelic imbalance of 20%/80% at the higher read counts, but at read counts less than 3000, there were instances where the software reported homozygous result. This suggests that when read count is relatively low, typing needs to be checked carefully at any time there appears to be an allele imbalance, or if the result is homozygous.

Based on these results, we believe that a considerably higher number of samples can be multiplexed per MiSeq run than the 36 samples used in this study while maintaining confidence in HLA typing assignments. Therefore, we found it informative to perform the down-sampling experiment with our own method to determine the minimum number of reads required per sample. In the limited number of samples that we studied, we found that having greater than 4000 reads per sample provided adequate coverage depth for confident HLA typing.

Discussion

One of the considerations when undertaking HLA genotyping by NGS is the up-front capital equipment cost of the sequencer. Four instruments have been reportedly used for HLA NGS typing: PacBio RSII, Roche 454, Thermo Fisher Ion Torrent and the Ilumina MiSeq. Each instrument has a number of advantages and disadvantages. The PacBio RSII's appeal is its ability to sequence full-length coding regions (29, 30) without the need for amplicon fragmentation or subsequent steps required for end-repair. Preliminary work shows promise in obtaining accurate class I allele calling (31). Much of the early work on HLA NGS genotyping was performed on the Roche 454 (26, 32–39); however, Roche will not be supporting this instrument after 2016. The Thermo Fisher Ion Torrent's most desirable feature is its short run time of 2 h although this run-time savings must first be preceded by a 5-h emulsion PCR step and several other labor-intensive steps to prepare the Ion chip for loading. More significantly, it is prone to errors, especially in homopolymer regions (40). A recent paper evaluating One Lambda's NGS workflow on the Ion Torrent reported excellent concordance, but noted that improvements could be made in coverage uniformity, allele balance, and read-length (41). Another report, comparing the MiSeq and the Ion Torrent (18), found that the MiSeq had a higher total output of bases and that each base had a higher quality score. Furthermore, the MiSeq had a higher fidelity rate when sequencing through the problematic homopolymer regions (18). For all of these reasons, we have selected the Illumina MiSeq as our instrument of choice. Its low error rate (40) coupled with its ability to perform read lengths up to 300 bps make it optimal for performing HLA sequencing. Further, the ability to sequence across introns and exons is important for highly polymorphic gene systems such as HLA and the resolution of cis/trans ambiguities. The primary disadvantage in choosing the MiSeq is its long run time of 40 h for the 2×250 read-length kit.

Essential to the actual DNA sequencing is the library construction process (42). All three methods evaluated here require the same general steps: long-range PCR, amplicon quantification, fragmentation, library preparation, size selection and a final quantification but there are significant differences in the details that will greatly affect the workflow in the clinical lab (Table 5). The hands-on-time is an important consideration. Owing to its many clean-up steps, the in-house method took the longest time with over 7 days required to process 24 samples. The Omixon Holotype ×2 24/7 and Illumina HLA TruSight v1 kits had the shortest hands-on-time with only 3 days. Because we performed all three methods manually and our experience was initially limited, the process may have taken a little longer than it would otherwise. Similarly, automation would greatly simplify all three methods, but will increase the equipment cost.

The clean-up steps are of particular concern. Amplicon clean-up is important for the removal of adaptor dimers or other DNA artifacts less than 100 bp in length. Removal of

these artifacts is essential because the small adaptor dimers or partial library constructs sequence very proficiently at the expense of the desired HLA target sequence (42, 43). Small-size artifacts will also affect quantification results. Efficient MiSeq cluster generation depends on loading the flow cell according to Illumina: over-loading results in poor template generation and low sequencing quality due to clusters being spaced too tightly together, whereas under-loading results in low data output and inefficient usage of the flow cell (43). The in-house method and the Illumina HLA TruSight v1 methods both require numerous library cleaning steps using either a combination of silica columns and AMPure beads or only AMPure beads. The in-house method had the highest number of clean-up steps, although, it may be possible to eliminate some of them with additional optimization. There are a number of disadvantages to the clean-up steps such as potential loss of DNA, increased labor time, and manual pipetting, and excessive pipette tip usage. The number of pipette tips required varies widely depending upon the method used. The Illumina v1 kit required the highest number of tips and was the most laborious to perform. This is due to its index-by-amplicon approach in contrast to the index-by-sample strategy employed by the Omixon and in-house methods. However, this approach would be quite suitable for single locus typing as required for disease association or drug hypersensitivity testing. The Omixon Holotype kit required the fewest consumables in that only two clean-up steps are required. The first is performed after pooling the amplicons for each gene for each sample, Exo-SAP-IT (Affymetrix) is added and the PCR plate is placed in the thermocycler. An additional clean-up step is performed after size selection when all 24 samples have been pooled, so the number of pipetting steps is markedly reduced. The complexity of library preparation for NGS is due to the number of individual steps as well as the number of individual tubes or wells required. In addition to the physical strains of manual pipetting, each sample and/or amplicon transfer has the potential for sample mix-up. Automation would mitigate these risks greatly but it can be costly; however, it is highly recommended depending on the testing volume for each individual laboratory.

HLA genotyping between the three methods were generally concordant at the second field. The exceptions were due to metric failures such as low Q30, low coverage, or a failure to type. There were a few instances using the Illumina Assign software where base pair or phasing mismatching prevented full allele genotyping. Moreover, Illumina's primer placement for HLA-DRB1 was responsible for 83% of the two-field ambiguity found with this method. Having access to more details and comparing primer placement for the commercially available kits would allow for a more accurate theoretical prediction of coverage and expected ambiguities for the available sequences. Moreover, only about 10% of all HLA alleles have been fully characterized from 5′UTR to 3′UTR in the IPD-IMGT/HLA Database (12). Given our limited data set and the large number of unknown alleles, it is likely that additional samples and allele combinations may reveal more ambiguities and discrepancies between the three methods, therefore, prior to clinical implementation, a full validation exercise should be conducted.

In summary, our data have shown that all three HLA NGS genotyping methods tested in this study performed well, although some were easier to perform than others. Overall, based on our experience presented here we favor assays that can provide the most accurate and unambiguous HLA allele assignments with the least amount of library preparation work and fewest transfer steps.

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Supporting Information

The following supporting information is available for this article:

Table S1. Results of 10 International Histocompatibility Working Group (IHWG) samples.

CHAPTER 4

CONCLUSION

The two peer-reviewed published manuscripts enclosed in this thesis, “Report on the effects of fragment size, indexing, and read length on HLA sequencing on the Illumina MiSeq” and “HLA genotyping in the clinical laboratory: Comparison of next-generation sequencing methods” represent research to establish that NGS is the new gold standard for HLA genotyping. We showed that HLA Genotyping by NGS produces more correct and unambiguous results than Sanger sequencing without the need for reflex testing.

NGS-based HLA typing presented here differs from Sanger sequencing in that the whole gene, or most of the gene, is sequenced. Routine clinical Sanger sequencing only interrogates exons 2-4 for class I, and exon 2 for class II. Table 4.1 compares regions sequenced between the three different methods.

The ability to sequence more introns and exons represents an improvement over Sanger sequencing and, consequently, provides greater allelic resolution than previously possible. As gaps in the IMGT database are filled, 3rd and 4th field allele resolution will be more readily attainable. The Omixon Holotype kit did not include DPA1 typing when it was initially evaluated but recent updates to the kit include primers for DPA1. No further work to accommodate additional loci has been done with the in-house kit.

Our conclusion in Chapter 2 regarding indexing strategy was vital in showing that NGS can be more streamlined. There, the index-by-amplicon and index-by-patient strategies were compared using the in-house method. Both methods are equal in providing correct and unambiguous typing results. It is noted in Chapter 3 that the Illumina HLA TruSight kit required an index-by-amplicon approach. Some problems associated with indexing-by-amplicon include increased usage of pipette tips and

Table 4.1. Comparison of HLA Exons/Introns Sequenced by NGS.

Target Sequence			
Loci	**Omixon Holotype X2 24/7**	**Illumina HLA TruSight**	**In-House**
HLA-A	Entire gene	Entire gene	Entire gene
HLA-B	Entire gene	Entire gene	Entire gene
HLA-C	Entire gene	Entire gene	Entire gene
HLA-DRB1	Intron 1-exon 5	Exon 2-intron 4	Intron 1-intron 4
HLA-DQB1	Entire gene (2 primer sets)	Entire gene	intron 1-intron 4
HLA-DPB1	intron 1-3'UTR	exon 2-3'UTR	Not tested
HLA-DQA1	Entire gene	Entire gene	Not tested
HLA-DPA1	Not tested	Entire gene	Not tested

Note: Results from genes not tested are not included.

physical strain due to manual pipetting. Furthermore, there is a heightened potential for sample mix-up, therefore, the index-by-patient technique is the preferred and more economical method.

The hallmark of NGS HLA genotyping is the achievement of high-resolution genotyping without the need for secondary testing. This is accomplished through quantification of clonal DNA libraries, phasing, and paired-end sequencing to maximize confidence of alignment to the reference sequence. This was demonstrated in chapters two and three. Resolution of cis/trans ambiguities requires that the correct sized DNA library be used with the appropriate MiSeq read-length kit. The average length of the HLA exons 2-4 is approximately 270 bp so it is advantageous to have an insert size larger than 270 bp in order to span across the exon and into the intron. For the in-house method, 100-300 bp and 300-600 bp fragment libraries were initially compared. Later, the limits of the in-house method's fragmentase enzyme were explored in order to create larger fragments. The constraints of AMPure beads for size selection required use of the PippinPrep for selection of the larger sized fragments. In addition, a no-size selection library was examined. The larger fragments compared included, 600-900 bp, 600-1200 bp, 900-1200 bp fragments. In the end, the 600-900 bp, PippinPrep size-selected library achieved the best resolution with 98% of all alleles typing correctly and unambiguously (Table 3.3).

The Illumina read-length kit is important in the discussion of fragment size. Three MiSeq reagent kits were compared in the two papers, 2x150, 2x250, and 2x300 bp. The 2x150 bp kit was only evaluated in the first paper with the smaller fragments. Use of larger fragment libraries with the smallest kit would be ineffective as the effort required

to create the larger fragments would not result in any significant benefit. However, with the longer fragments, the 2x250 read-length kit, and the use of the paired-end sequencing kits, distant polymorphisms can be spanned and complete phasing of the gene is accomplished. Surprisingly, the larger 2x300 kit did not improve the overall correct and unambiguous typing rate. It also required an additional 12 hours on the MiSeq instrument in comparison to the 2x250 kit. Therefore, the 2x250 read-length kit was selected as the optimal read-length kit for the in-house method.

Commercial HLA genotyping by NGS kits were not available at the time of initial research and development of the in-house method but later were released. This development led to the comparison in Chapter 3 of two commercial kits, the Omixon 24/7 Holotype and the Illumina HLA TruSight kits. Both commercial assays were easy to execute but results were mixed (see Table 3.4). Briefly, the Illumina HLA TruSight kit had the poorest performance with only 82.5% of all samples typing correctly and unambiguously for the second field. Much of the poor performance is due to ambiguous DRB1 results. This is due to the location of the forward primer. Its placement, inside of the start of exon 2, prevents resolution of alleles that only differ in that position. The Omixon Holotype X2 24/7 kit performed well with 95.6% of all samples typing correctly and unambiguously but the in-house method performed the best. There, 97.6% of all samples typed correctly and unambiguously.

An important metric for quality is the depth of coverage because redundancy in sequencing ensures confidence in alignment to the reference sequence. Average coverages were between 70 and 697 for all three methods (Table 3.6). DRB1 for the Omixon kit had the most problems with low average coverage with 10.4% of all sample

having low coverage. The Omixon Twin software flags any sample with less than 25 copies per base, suggesting that these samples be further investigated. The in-house method had the highest overall coverage of 697. This suggests that more patient samples could be added to flow cell making the assay more cost effective without sacrificing sequencing quality.

An important consideration in kit selection is the workflow of the assay. As previously stated, the efficacy of the index-by-patient approach was demonstrated by our studies in Chapter 2, yet the Illumina HLA TruSight kit still required the index-by-amplicon approach. This resulted in extra time, effort, and supplies. The in-house method, despite the adoption of the index-by-patient strategy, has some drawbacks to its workflow. Specifically, the number of clean-up steps required after each step of the library preparation process. A consequence of the increased number of clean-up steps is the potential loss of DNA, increased labor time, manual pipetting, and increased pipette tip usage. However, it is possible that this assay could be further optimized and the number of clean-up steps reduced. Therefore, at this stage, the Omixon Holotype X2 24/7 kit had the best performance with the easiest workflow to implement for clinical testing.

We showed that HLA Genotyping by NGS produces more correct and unambiguous results than Sanger sequencing without the need for reflex testing. Over 97% of all samples were typed correctly and unambiguously without the need for reflexive testing using the in-house method. Follow-up studies will include the evaluation of newer commercial kits as well as testing more HLA alleles.