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Performance Characteristics and Validation of Next-Generation Sequencing for Human Leucocyte Antigen Typing

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High-resolution human leukocyte antigen (HLA) matching reduces graft-*versus*-host disease and improves overall patient survival after hematopoietic stem cell transplant. Sanger sequencing has been the gold standard for HLA typing since 1996. However, given the increasing number of new HLA alleles identified and the complexity of the HLA genes, clinical HLA typing by Sanger sequencing requires several rounds of additional testing to provide allele-level resolution. Although next-generation sequencing (NGS) is routinely used in molecular genetics, few clinical HLA laboratories use the technology. The performance characteristics of NGS HLA typing using TruSight HLA were determined using Sanger sequencing as the reference method. In total, 211 samples were analyzed with an overall accuracy of 99.8% (2954/2961) and 46 samples were analyzed for precision with 100% (368/368) reproducibility. Most discordant alleles were because of technical error rather than assay performance. More important, the ambiguity rate was 3.5% (103/2961). Seventy-four percentage of the ambiguities were within the *DRB1* and *DRB4* loci. HLA typing by NGS saves approximately $6000 per run when compared to Sanger sequencing. Thus, TruSight HLA assay enables high-throughput HLA typing with an accuracy, precision, ambiguity rate, and cost savings that should facilitate adoption of NGS technology in clinical HLA laboratories. *(J Mol Diagn 2016, 18: 668–675; http://dx.doi.org/10.1016/j.jmoldx.2016.03.009)*

It is widely accepted that human leukocyte antigen (HLA) matching reduces patient morbidity and mortality after hematopoietic stem cell transplantation (HSCT).[1] The current standard of care is high-resolution HLA typing by Sanger sequencing or sequence-based typing (SBT). High-resolution HLA typing is defined as a set of alleles that specifies and encodes the same protein sequence for the peptide-binding region of an HLA molecule. However, SBT cannot accurately phase heterozygous alleles and provides limited sequencing information. Traditionally, HLA typing by SBT typically involves sequencing only exons 2, 3, and 4 of HLA class I genes and exons 2 and 3 of HLA class II genes. Since the regulatory requirement was established for high-resolution HLA typing for *HLA-A/B/C/DRB1* in 2005, many clinical laboratories are putting significant resources toward ambiguity resolution.[2] Even as several restrictions were removed, such as the requirement to exclude rare alleles, there is still a growing list of ambiguities that require additional testing and delay patient results. The issue of ambiguities is a testament to the complexity of the HLA region in the human genome with >13,000 alleles identified to date.[3] The combination of the inability to phase heterozygous alleles and the growing number of HLA alleles has led to a significant number of ambiguities in HLA typing that require time-consuming and costly additional tests to be performed. In 2004, Adams et al[4] reported the ambiguity rate for *HLA-A*, *HLA-B*, and *HLA-C* of 24% to 41%. Three years later, Voorter et al[5] reported that ambiguities for *HLA-A*, *HLA-B*, and *HLA-C* had increased to approximately 50%.

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Currently, our experience is that ambiguity resolution is required in 53% of patient specimens to determine a specific allele (E.T. Weimer and J.L. Schmitz, unpublished observation). This high level of additional testing delays patient HLA typing results and increases the cost of HLA typing. With no sign that the number of HLA alleles identified will decline and the increasing application of typing of additional loci (*DPB1* and *DRB3/4/5*), there is a great need for a technology that allows for accurate high-resolution HLA typing without the requirement of additional testing.[3,6–8]

Next-generation sequencing (NGS) technology is the massive parallel sequencing of clonal DNA molecules. By using unique molecular signatures or barcodes, many samples can be pooled together and sequenced simultaneously. A key feature of the NGS method is the ability to generate massive amounts of genetic data from many DNA molecules simultaneously.[9,10] The combination of clonal DNA sequencing and application of long-range PCR techniques to increase HLA genomic information aids in the reduction in HLA allele ambiguities. For example, Danzer et al[11] used long-range PCR of HLA genes and NGS to demonstrate an average ambiguity reduction of 93.5% for *HLA-A*, *HLA-B*, *HLA-C*, *DRB1*, *DQB1*, and *DPB1*. Although the ambiguity reduction varied by locus [ie, the reduction of *DRB1* was less pronounced (46.1%)], this was still considered significant given the amount of additional testing required by SBT.

HLA typing by NGS using long-range PCR techniques to increase genomic coverage of HLA genes has proven effective at improving ambiguity resolution.[11–14] The increased genomic coverage is partly what enables the accurate phasing of heterozygous bp positions often observed in SBT.[13,15] Several recent reports have demonstrated the feasibility of using NGS technology to provide >97% concordance with SBT.[11,13,15–17] Although the high-throughput nature of NGS is thought to be cost-effective for HLA typing, this has yet to be shown. More important, previous reports focused on laboratory-developed assays rather than commercially available reagents.[11–14,17,18] With the increasing availability of commercial NGS HLA reagents, there is a need to better understand each assay's characteristics and their utility to solve the limitations of SBT.

In this study, we evaluated the performance of the TruSight HLA assay, a commercially available NGS assay for HLA typing, to not only accurately type HLA alleles but also identify a set of quality control criteria required to ensure accurate HLA allele determination. More important, we also performed a cost analysis between NGS and SBT for HLA typing that provides the first evidence that NGS is a cost-effective alternative to SBT.

Materials and Methods

Patient Samples, DNA Extraction, and HLA Gene Amplification

Two-hundred and eleven samples that were already high-resolution HLA typed were used for comparison and clinical validation. An additional 79 samples of genomic DNA extracted from buccal swabs from patients and donors under evaluation for HSCT were also used. Genomic DNA was extracted from each sample using Qiagen DNA Tissue Extraction kits (Qiagen, Valencia, CA). The study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

For HLA gene amplification, primers specific for each HLA gene were used in a long-range PCR. DNA was quantified using a QuBit fluorometer (Life Technologies, Carlsbad, CA). After quantification, sample DNA was diluted to 10 ng/μL. For buccal swabs, 40 of the 79 samples received additional purification according to the manufacturer's instructions. Fifty nanogram of genomic DNA was used for each HLA locus and amplified according to the manufacturer's instructions. PCR amplicons were visualized using 2% agarose gel electrophoresis before preparing NGS libraries. Twenty-four samples (192 HLA loci) were run in a single NGS experiment. A sample with a known HLA typing was run on each NGS run to ensure library preparation, data quality, and analysis were of sufficient quality to ensure accurate HLA typing.

TruSight HLA Library Preparations and NGS Sequencing

Amplified DNA for NGS sequencing was prepared according to the supplied instructions (Illumina, Inc., San Diego, CA). Briefly, amplified DNA was purified using magnetic AMPure XP beads, fragmented, and Illumina-specific adaptors were applied using a tagmentation enzyme supplied by Illumina. After tagmentation, fragments were purified using AMPure XP beads and patient-specific indices were added to individual HLA loci by a short PCR, followed by magnetic bead purification. Post-barcoding samples were pooled and the libraries quantified using QuBit fluorimeter. The size of HLA libraries was determined using TapeStation Bioanalyzer 2200.

To assess sequencer-based errors, a 1% to 5% concentration of 12.5 pmol/L PhiX control (Illumina, San Diego, CA) was spiked into pooled HLA libraries. Pooled HLA libraries and PhiX control were loaded onto the cartridge and 2 × 250 bp sequencing was performed using a regular flow cell on an Illumina MiSeq. Demultiplexing and generation of FASTQ files was performed on the MiSeq system. A total of 34 MiSeq runs were performed and used for quantification of sequencing metrics.

Sample Analysis

Sample analysis was performed using Conexio Assign for TruSight HLA software version 1.0.0.729 supplied by Illumina. Sample consensus sequences were compared to the IMGT/HLA database (version 3.15 to 3.20). A complete HLA genotype was determined by loading FASTQ files into Conexio Assign software. Samples were analyzed for mismatches throughout the entire amplified region of the HLA

genes. Mismatches that represented potential novel HLA alleles were noted. Ambiguity resolution was performed by Sanger sequencing with analysis on uTYPE 6.0 (SeCore HLA kit; Life Technologies).

Cost Analysis

The cost analysis between SBT and NGS was performed using the available list price for each reagent necessary for the respective technologies. For NGS, reagents included HLA locus−specific primers, library preparation, and sequencing reagents. For Sanger sequencing, reagents included HLA locus−specific primers, ExoSAP, sequencing reagents (cathode, anode, polymer), sequence-specific oligonucleotides, and group-specific sequencing primers for ambiguity resolution. Ambiguity resolution was added to each technology using laboratory-specific 53% for Sanger sequencing and 3% for NGS. Instrumentation cost was excluded for the analysis. An estimation of time required for each method was determined by averaging the hands-on time from amplification to completed analysis (including ambiguity resolution) for each technologist ($n = 5$) performing the assay. The average labor time was multiplied by the average hourly rate for HLA technologists at UNC Hospitals. Assay time was defined as the time from PCR to completion of sequencing analysis. Turnaround-time (TAT) was determined for all HSCT-related HLA typings from receiving in laboratory to verification of results in calendar days. Date range for NGS ($n = 323$) was August to December 2015 and the same time period 1 year prior for Sanger sequencing ($n = 324$). NGS runs were between 10 and 23 patients per run, with *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DRB1*, *HLA-DQB1*, *HLA-DQA1*, *HLA-DPB1*, *HLA-DPA1* typed. Only *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DRB1*, *HLA-DQB1*, *HLA-DPB1* were reported clinically. *HLA-DQA1* and *HLA-DPA1* typing were used for academic purposes. For comparison, a maximum of five patients were run using Sanger sequencing.

Statistical Analysis

Significant differences between two proportions were determined by two-tailed probability test from the calculated z-ratio. Significant differences between NGS and Sanger for TAT were calculated using unpaired *t*-test with Welch's correction. $P \leq 0.05$ was considered significant.

Results

HLA Gene Amplification Rates

To enrich HLA genes, genomic DNA was amplified by long-range PCR. A known HLA-typed sample and negative control were used with every PCR to ensure proper HLA amplification and to assess for potential contamination. There were no cases of PCR-based contamination throughout the validation. The PCR fragment length for each HLA gene is shown in Table 1, and a representative gel electrophoresis of HLA amplicons is shown in Figure 1A. Overall, the amplification success rate of HLA genes was 95.0% (2399/2526) (Figure 1B). Only 37 HLA class I genes failed to amplify of 945 amplifications (3.9%) and 31 of the 37 were from buccal swabs. There were a total of 127 incidences of no detectable PCR band on electrophoresis that were subsequently sequenced and HLA typed (Figure 1, C and D). *DPA1* and *DPB1* were the most difficult HLA genes to amplify, most likely because of their length (Figure 1, B−D). In addition, *DPA1* and *DPB1* had the lowest HLA typing success overall. Amplification success was significantly lower for *DPA1* ($P < 0.001$), *DPB1* ($P = 0.001$), and *DQB1* ($P = 0.006$) when using DNA isolated from buccal swabs (BSs) compared to DNA from peripheral blood[19] (Figure 1B). To determine the reason for decreased HLA amplification rate from BSs, the effect of concentration and quality of DNA on HLA typing was evaluated. Additional purification of BSs significantly increased HLA typing rates for *DQB1* ($P = 0.048$) and DPB1 ($P = 0.002$) compared to nonpurified samples (Figure 1E). Similarly, BSs with higher concentrations were significantly more often successfully HLA typed for *DPA1* ($P < 0.001$) and *DPB1* ($P = 0.001$) compared to lower concentrations (Figure 1F).

Characteristics of HLA Library Fragments and NGS Data

NGS library fragments vary in size and DNA fragments >1000 bp are less efficient at cluster generation on the MiSeq than smaller fragments.[19] To determine the DNA fragment size generated using TruSight HLA, three (576 loci) pooled HLA libraries were analyzed using a TapeStation Bioanalyzer. The average library fragment size was 1268 bp (95% CI, 856−1680 bp) (Figure 2A). Larger fragment sizes aid in correct phasing of HLA alleles.[15] Next, overall read quality and depth of coverage were determined for each HLA locus. The average percentage of reads ≥ Q30 was 96.1% (95% CI, 95.4%−96.7%) for all HLA loci (Figure 2B). The lowest quality reads were consistently observed with *HLA-B* and *HLA-DQB1*. The average depth of coverage was 280 (95% CI, 270−289) for

Table 1 Amplification Size for HLA Genes Using TruSight HLA

Loci	Sequencer region (kb)
A	4.1
B	2.6
C	4.2
DPA1	10.3
DPB1	9.7
DQA1	7.3
DQB1	7.1
DRB1/3/4/5	4.1

HLA, human leucocyte antigen.

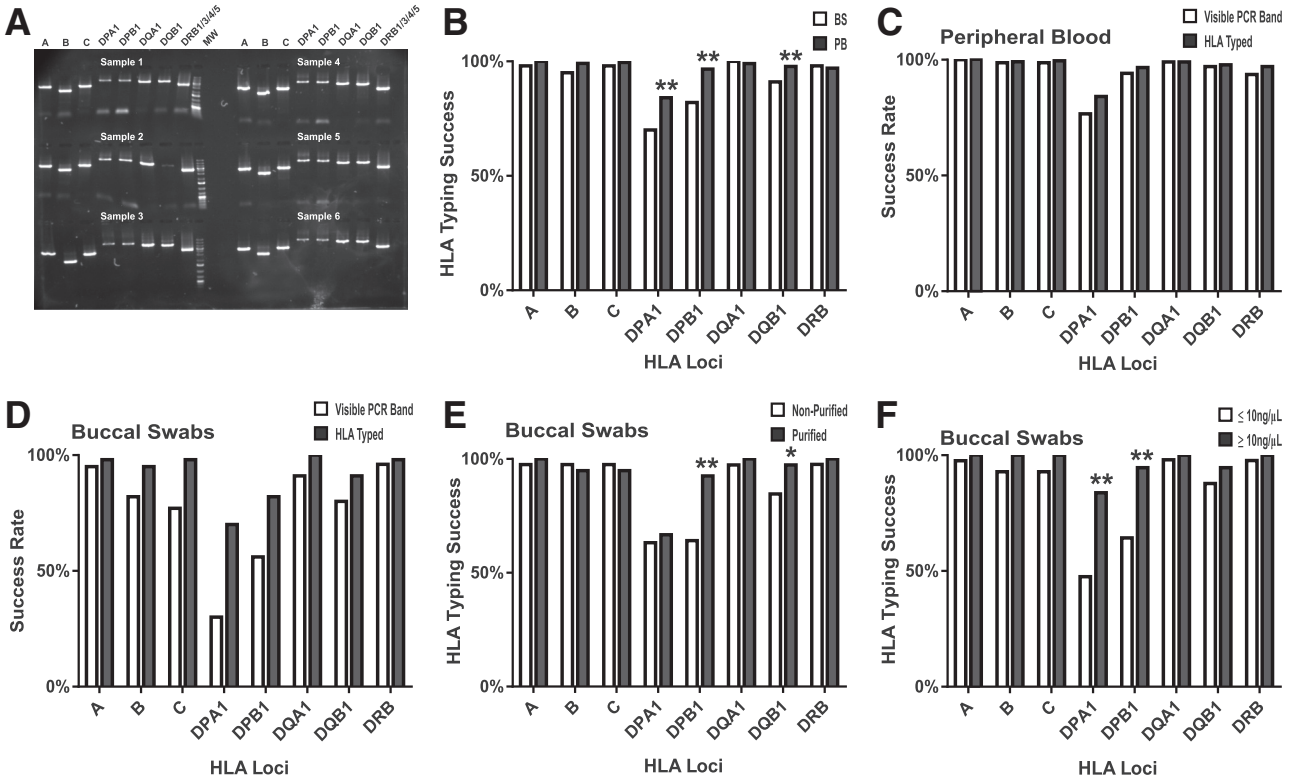

Figure 1 HLA typing rates for peripheral blood and buccal swabs. **A:** Representative gel electrophoresis of TruSight HLA amplicons. **B:** Comparison between peripheral blood (PB) and buccal swab (BS) HLA successful typing rates. **C:** Peripheral blood. **D:** Buccal swabs. **C** and **D:** Absence of a visible electrophoresis band does not prevent accurate HLA typing. Gel electrophoresis bands were counted and compared to the overall ability of each sample to accurately HLA type. **E:** Additional purification of buccal swabs provides better HLA typing. HLA typing was compared between buccal swab samples that were either used as extract (nonpurified) or received additional magnetic bead-based purification (purified). **F:** Higher concentration buccal swabs outperform low concentration samples. $*P < 0.05$, $**P < 0.005$.

all HLA loci (Figure 2C). These results indicate that NGS data generated by TruSight HLA are high quality and core exons within each HLA are covered beyond 250×.

NGS Data Quality Metrics

To determine the quality of each library preparation and sequencing run, cluster density, percentage of clusters passing filter, percentage of reads ≥ Q30, and error rate were monitored using Illumina's sequencing analysis viewer. The acceptable range for each parameter was determined to be the average plus or minus 2 SDs or using the MiSeq performance specifications provided by the manufacturer. There were two runs in which PhiX was not added to the pooled libraries before sequencing and thus no error rate could be determined. The shaded areas in Figure 3 show the acceptable range for each parameter. For a run to be considered high quality, it must fall within specific ranges for each category. There were two runs that required repeat sequencing because of reagent issues (Figure 3, B

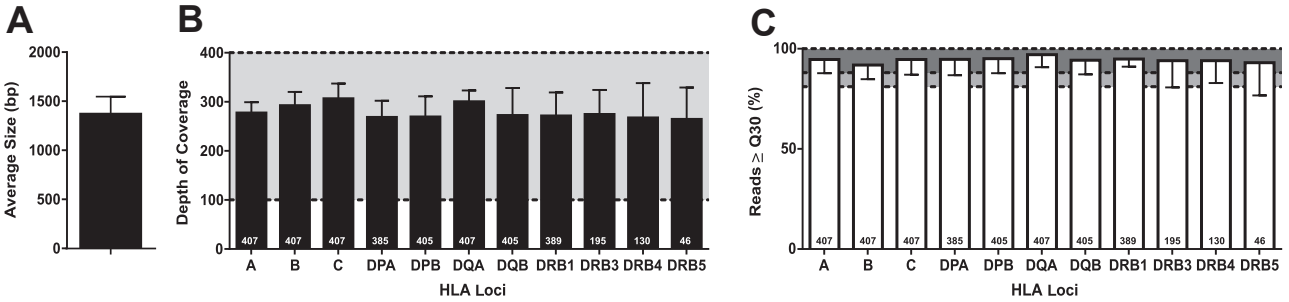

Figure 2 Characteristics of next-generation sequencing (NGS) library fragments. **A:** NGS library fragment sizes generated using TruSight HLA. Library fragment size was determined from pooled libraries using a TapeStation Bioanalyzer. **B:** Percentage of reads ≥ Q30. **Shaded area** indicates acceptable range. **C:** Depth of coverages for each HLA loci. **Light gray area** indicates acceptable range for HLA-A, HLA-B, HLA-C, HLA-DPA1, HLA-DPB1, HLA-DQB1, and HLA-DQA1. **Dark gray area** indicates acceptable range for HLA-DRB1/3/4/5. **B** and **C:** The number of individual measurements is indicated within each column. Data are expressed as means ± SD (**A**–**C**). $n = 3$ (**A**).

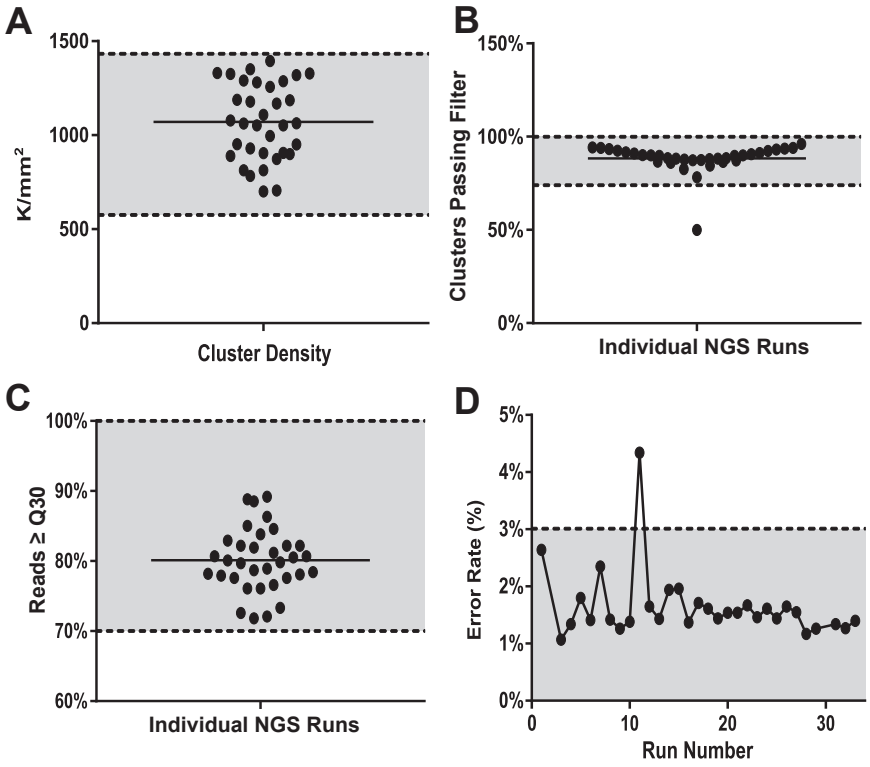

Figure 3 Characterization of next-generation sequencing (NGS) data metrics. For 34 NGS runs, cluster density, frequency of clusters passing filter, percentage of reads ≥ Q30, and error rate were determined. All of the parameters were determined using Sequence Analysis Viewer (SAV) after each run. Error was determined by spiking in 1% to 5% PhiX into pooled HLA libraries before sequencing. **Gray regions** indicate acceptable range for each parameter. **A**–**C:** Line indicates mean value. **A:** Cluster density. **B:** Clusters passing filter. **C:** Frequency of reads at or above Q30. **D:** PhiX error rate. Two separate sequencing runs fell outside acceptable range because of reagent issues.

and D). The average cluster density and proportion of clusters passing filter were 1071 ± 35 K/mm^2 and 88% ± 1%, respectively (Figure 3, A and B). The average percentage of read ≥ Q30 was 80% ± 1% (Figure 3C). The average error rate was 1.65% ± 0.1% (Figure 3D). After run 7, there was a 54.5% reduction in error rate variability compared to the first six runs (Figure 3D). Establishing these criteria is crucial for ensuring library preparation consistency and sequencing performance over time.

HLA Typing Comparison between Sanger and NGS

Clinical validation of HLA typing by NGS involves determination of the assay's accuracy and precision compared to the gold standard, Sanger sequencing. To determine TruSight HLA assay accuracy, 211 samples for which existing genomic DNA and high-resolution HLA typing was known for each locus were used. Fifty of the 211 samples were blinded (all authors were blinded). Forty-six samples were used to assess assay reproducibility. HLA alleles were considered equivalent on the basis of the National Marrow Donor Program HLA reporting criteria. The National Marrow Donor Program requires identification of eight null (nonexpressed) HLA alleles within specific HLA G groups.[20] All eight null HLA alleles could be identified or ruled out as potential HLA alleles using TruSight HLA. Overall, accuracy for the assay was (2954/2961) 99.8% with a precision of 100% (Table 2). Two-hundred and sixty-five unique HLA alleles were accurately identified by TruSight HLA (Supplemental Table S1). Only ambiguities outside of HLA G groups are considered significant because those alter the peptide-binding region of the HLA protein. There were 103 (3.5%) ambiguities of 2961 alleles identified. Of 103 ambiguities, 91 (88.4%) were from *DRB1* (26.3% of all *DRB1* typings) and 11 (10.7%) were from *DRB4* (47.8% of all *DRB4* typings) loci (Table 2). Because *DRB4* is not commonly part of HLA matching for HSCT, the impact on routine clinical use is minimal. There was one ambiguity in

Table 2 Accuracy and Ambiguities for TruSight HLA Assay

Gene	*N*	Allele level mismatch	% correct	Ambiguities	Ambiguity rate (%)
HLA-A	353	1	99.7	1*	0.3
HLA-B	353	1	99.7	0	0.0
HLA-C	353	0	100.0	0	0.0
HLA-DPA1	345	1	99.7	0	0.0
HLA-DPB1	354	0	100.0	0	0.0
HLA-DQA1	346	1	99.7	0	0.0
HLA-DQB1	345	0	100.0	0	0.0
HLA-DRB1	346	1	99.7	91†	26.3
HLA-DRB3	100	0	100.0	0	0.0
HLA-DRB4	23	0	100.0	11‡	47.8
HLA-DRB5	43	1	97.7	0	0.0
Total	2961	6	99.8	103	3.5

*Ambiguity exists between 03:01:01, 11:01:01 pair, and 03:63/11:12.

†Ambiguities exist for the following HLA alleles: 03:01; 03:50, 08:04; 08:59, 15:01; 15:110, 13:01; 13:112, 16:02; 16:22, 14:54; 14:113; 14:125; 14:157, 15:02; 15:19, 13:02; 13:128.

‡Ambiguities because of inability to accurately identify 01:03N.

the *A* locus (0.3%). All remaining HLA loci had no ambiguities. HLA typing by TruSight HLA demonstrated a 93.4% reduction in ambiguities compared to Sanger sequencing.

The seven cases of incorrect HLA typing emphasized the need for HLA-specific data criteria. Three of the seven instances were because of allele dropout. Given those results, HLA-specific data quality criteria were established. A depth of coverage (DOC) of at least 100 and at least 81% of the reads $\geq$ Q30 were determined to be necessary for *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DRB1*, *HLA-DQB1*, *HLA-DQA1*, *HLA-DPB1*, *HLA-DPA1* typing. Higher quality data (88% of the reads are $\geq$ Q30) was necessary for *HLA-DRB3/4/5* to prevent false identification of those alleles (Figure 2, B and C). The sequencing and HLA-specific data quality metrics were validated using the 50 blinded samples. Although the above criteria are crucial for increased accuracy in HLA typing results, samples could be accurately typed below those thresholds. Samples were accurately typed with as few as 67 reads and a 74% frequency of $\geq$ Q30 reads (data not shown). Higher DOC was a better HLA typing quality predictor compared to quality scores (Table 3). Of note, if a sample meets the sequencing run parameters but fails HLA-specific criteria, HLA typing for that sample is not reported and must be repeated. More important, repeat amplification and NGS library preparation accurately typed all incorrect samples, suggesting the incorrect HLA typing was because of a technical error issue rather than an assay issue (Table 3).

HLA Typing Cost Comparison between Sanger Sequencing and NGS

As mentioned above, 53% of the HLA typings by Sanger sequencing require additional tests to resolve ambiguities in our laboratory. Ambiguity resolution delays patient results and increases patient cost for HSCT. Given that there was significant reduction in the number of ambiguities encountered by TruSight HLA, we hypothesized that NGS sequencing would be more cost effective than typing by Sanger sequencing. The relative cost of HLA typing by Sanger sequencing and TruSight HLA was determined and compared. For each technology, all reagent costs and relative hands-on time were considered. The average time for an assay and hourly rate of the technologists were used to calculate labor costs. TruSight HLA reagent costs were 41.8% ($270/patient) lower compared to Sanger sequencing for HLA typing at all loci (Figure 4A). There was a 25% ($5/patient) reduction in labor (library preparation and analysis) required for TruSight HLA compared to Sanger sequencing. High-resolution HLA typing data for *HLA-A/HLA-B/HLA-C/HLA-DRB1/HLA-DQB1* costs between NGS and Sanger were determined. The total cost to type those HLA loci by NGS is approximately $250 ($50/HLA locus) per patient and $380 ($76/HLA locus) per patient for Sanger sequencing (Figure 4B). Thus, the more patients run together for NGS the more cost savings that are realized. To examine the impact of batching samples TAT for HLA typing by NGS was compared to Sanger sequencing during the same time period. The average TAT using NGS was significantly faster (19 days, $P < 0.001$) compared to Sanger sequencing (22 days) (Figure 4C). Thus, in addition to being highly accurate, TruSight HLA is cost-effective while not delaying patient HLA typing.

Table 3 Quality Metrics on Incorrectly Typed Samples

ID*	Average % of read $\geq$ Q30	Average DOC	HLA typing
84	93	30	DRB3 present
Repeat†			No DRB3 present
141	91	77	DRB1*15:01
Repeat†	97	293	DRB1*12:01G
UNC9			DQB1*06 dropout
Repeat†	96	277	DQB1*06:02
122	94	49	DRB3 present
Repeat†			No DRB3 present
2417			DQB1*06 dropout
Repeat†	95	277	DQB1*06:02
4609			DQB1*06 dropout
Repeat†	96	284	DQB1*06:03
UNC7			DRB1*04 dropout
Repeat†	89	164	DRB1*04:04

*All incorrect samples were flagged by the Conexio Assign for TruSight HLA software.

†All repeat testing was consistent with Sanger sequencing results.

DOC, depth of coverage; HLA, human leucocyte antigen.

Discussion

The analysis presented herein is not only the first characterization and validation of the TruSight HLA assay for HLA typing by NGS, but is also the first report of specific sequencing and HLA criteria necessary for clinical reporting of HLA results. TruSight HLA demonstrated efficient generation of long-range HLA amplicons (Figure 1) and NGS libraries that consistently generated high-quality data (Figures 2 and 3). The degree of concordance is consistent with previous reports comparing different sequencing platforms and NGS HLA assays[17,21,22] (Table 2). The cost-effectiveness of HLA typing by NGS is consistent with a report by Stoddard et al[23] on the use of NGS compared to Sanger for diseases with multiple candidate genes (Figure 4). Overall, TruSight HLA is a robust assay that consistently provides accurate high-resolution HLA typing with a dramatically reduced ambiguity rate, cost, and TAT.

Clinical implementation of new HLA typing technology requires strict validation to provide HLA typing for patients. There are few clinical HLA laboratories performing HLA typing by NGS compared to Sanger sequencing. Establishing criteria for what should be considered sufficient validation is a vital part of the process for clinical laboratories. Several of the NGS runs experienced reagent

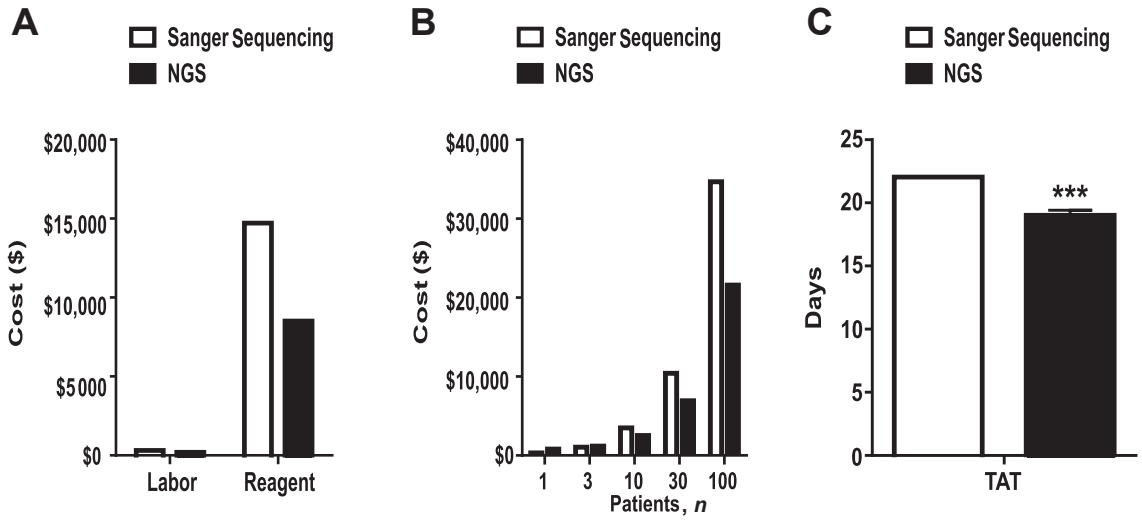

Figure 4 HLA typing cost comparison: next-generation sequencing (NGS) and Sanger sequencing. **A:** Cost analysis between NGS and Sanger sequencing was performed using list prices for reagents and the mean labor time for amplification, library preparation, and analysis. The cost is based on NGS runs of 23 patients per run for all HLA loci. Ambiguity rate for Sanger sequencing (53%) and NGS (3.5%) were factored into final reagent and labor costs. **B:** Cost for typing HLA-A/HLA-B/HLA-C/HLA-DRB1/HLA-DQB1 for various numbers of patients between NGS (black bars) and Sanger sequencing (white bars). **C:** Turn-around-time (TAT) between Sanger (2014) sequencing and NGS (2015) for August to December. $n = 5$ (**A**); $n = 323$ (**C**, 2015); $n = 324$ (**C**, 2014). $^{***}P < 0.001$.

issues that highlighted the need to establish guidelines for an acceptable sequencing run to proceed to HLA analysis (Figure 3, C and D). This report demonstrates one such manner of approaching the issue. Without the criteria presented herein, the accuracy of HLA typing would have been significantly decreased. In addition, clinical laboratories must have a quality assurance system in place to monitor assay performance. More important, DOC was a better indicator for data quality than quality score (Table 3). Higher quality scores were associated with less phasing issues for the HLA region and thus incorporated for quality assurance measures (data not shown). Tracking the sequencing metrics meets this clinical laboratory standard (Figure 3). As an additional level of quality control, HLA-specific data quality criteria were determined (Figure 2). Application of these criteria allowed for increased accuracy of HLA typing and reduced the potential for false-positive HLA typings. In our experience, failed HLA typing is most often because of poor sample quality (Figure 1) or improper handling of the magnetic beads (M. Montgomery and E.T. Weimer, unpublished observation). Laboratories considering NGS for HLA typing should examine their workload and the TAT wanted. Thus far, NGS has improved the TAT for high-resolution HLA typing, this will be dependent on the frequency of samples the laboratory receives and the TAT wanted. The cost benefit of NGS is greatly diminished as the batching of samples is reduced (Figure 4C).

A thorough validation should not only determine whether an assay performs robustly, but also identify areas of concern. Consistent with previous reports, HLA typing of *DRB1/3/4/5* was the most difficult[22,24,25] (Table 2). These issues arise because of primer locations inhibiting the ability to resolve certain HLA alleles. All instances where the software inappropriately identified *DRB3/4/5* alleles had DOC of <80. After application, the proposed HLA-specific criteria (Figure 2, B and C), those samples would have been repeated before reporting results. A known issue with PCR amplification methods is the potential for allelic drop out. *DQB1**06 (5% of *DQB1**06 typings), *DRB1**04 (2.9% of total *DRB1**04 typings), and *B**27:05:02 (low amplification rate) were found to be most susceptible to allelic drop out. This occurred most often from low DNA concentration buccal swabs and additional purification of buccal swabs before PCR significantly reduced allele drop out (M. Montgomery and E.T. Weimer, unpublished observations). Although several reports have demonstrated that cluster generation and MiSeq sequencing are less efficient with long DNA fragment sizes,[19,26] we and others have clearly demonstrated that DNA fragments >1000 bp can effectively and reproducibly generate high-quality NGS data using MiSeq (Figures 2 and 3).[15] More work is needed to improve HLA typing of *DQB1*, *DRB1*, and *DRB4* to further reduce ambiguities and cost. In addition, more time is needed to examine the impact transitioning to HLA typing by NGS will have on patient care and the utility of whole gene sequencing.

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Supplemental Data

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