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ORIGINAL ARTICLE

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Comparison of sequence-specific oligonucleotide probe vs next generation sequencing for HLA-A, B, C, DRB1, DRB3/B4/B5, DQA1, DQB1, DPA1, and DPB1 typing: Toward single-pass high-resolution HLA typing in support of solid organ and hematopoietic cell transplant programs

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Many clinical laboratories supporting solid organ transplant programs use multiple HLA genotyping technologies, depending on individual laboratory needs. Sequence-specific primers and quantitative polymerase chain reaction (qPCR) serve the rapid turnaround necessary for deceased donor workup, while sequence-specific oligonucleotide probe (SSOP) technology is widely employed for higher volumes. When clinical need mandates high-resolution data, Sanger sequencing-based typing (SBT) has been the "gold standard." However, all those methods commonly yield ambiguous typing results that utilize valuable laboratory resources when resolution is required. In solid organ transplantation, high-resolution typing may provide critical information for highly sensitized patients with donor-specific anti-HLA antibodies (DSA), particularly when DSA involve HLA alleles not discriminated by SSOP typing. Arguments against routine use of SBT include assay complexity, long turnaround times (TAT), and increased costs. Here, we compare a next generation sequencing (NGS) technology with SSOP for accuracy, effort, turnaround time, and level of resolution for genotyping of 11 HLA loci among 289 specimens from five clinical laboratories. Results were concordant except for SSOP misassignments in eight specimens and 21 novel sequences uniquely identified by NGS. With few exceptions, SSOP generated ambiguous results while NGS provided unambiguous three-field allele assignments. For complete HLA genotyping of up to 24 samples by either SSOP or NGS, bench work was completed on day 1 and typing results were available on day 2. This study provides compelling evidence that, although not viable for STAT typing of deceased

donors, a single-pass NGS HLA typing method has direct application for solid organ transplantation.

KEYWORDS
HLA genotyping, next generation sequencing, solid organ transplantation

1 | INTRODUCTION

Although it is widely accepted that HLA matching is associated with solid organ graft survival,[1] definition of an acceptable mismatch continues to evolve in concert with the ongoing evolution of technologies for HLA typing and for detection of donor-specific antibodies (DSA). Beyond donor-recipient HLA matching, solid organ compatibility determination involves assessment of recipient sensitization to HLA antigens. The impact of donor-specific antibodies (DSA) on organ survival has been well established,[2] and HLA alloantibody screening has long been routinely performed in clinical laboratories supporting solid organ transplantation. A paradigm shift, however, occurred with the advent of highly sensitive solid-phase antibody testing methodologies, and their routine use has revealed that patients can and do generate allele-specific antibodies.[3] This in turn has led to a shift in HLA typing from the traditional serology defined HLA antigens for solid organ donor selection to recognition of the need for higher-resolution HLA typing, particularly for highly sensitized patients. Duquesnoy et al[4] discuss the positive impact of high-resolution typing in highly sensitized patients awaiting living donor transplants. Kishikawa et al[5] argue that HLA class II eplet mismatching is a risk factor for de novo DSA and antibody-mediated rejection (AMR). Kamoun et al[6] reported that amino acid mismatches within the DRB1 antigen recognition site accounting for additional graft failure risk beyond the two-digit antigen level, and other studies show that DSA may result from mismatches for specific HLA alleles that may not be discriminated by low-resolution typing.[7,8]

Despite the mounting evidence that high-resolution HLA typing can benefit patient care, the accepted standard for solid organ transplant compatibility determination remains antigen level HLA typing due largely to the perceived challenges of implementing a complex new technology with potentially increased expenses and extended turnaround time (TAT) to reporting.[9] Currently, SSP (sequence-specific primers), sequence-specific oligonucleotide probe (SSOP), and quantitative polymerase chain reaction (qPCR) dominate the commercial kit market for intermediate-resolution HLA genotyping. SSP and qPCR provide the rapid TAT necessary for deceased donor workup but are less suited for large numbers of samples. The widely used SSOP technology, with probes bound to color coded microspheres and analysis on a flow cell instrument, provides 1- to 2-day TATs, depending on volume. When high-resolution typing is clinically necessary, Sanger SBT has hitherto been the gold standard despite the frequent ambiguous results that engender added time and expense for resolution.

Here, we compare a next generation sequencing (NGS) technology with SSOP for accuracy, effort, turnaround time, and level of resolution for genotyping of 11 HLA loci among 289 specimens from five clinical laboratories. While initial NGS systems involved cumbersome 4- to 5-day procedures,[9-13] the NGS kit used in this study allowed typing to be completed with a 2-day TAT, with all bench work and sequencer instrument loading accomplished on day 1. Compared with SSOP, this NGS protocol decreased use of consumables, reduced tech-to-tech variability, and increased the level of typing resolution at all loci. These considerations, together with equivalent costs of reagents for NGS vs SSOP, provide a compelling argument for the consolidation of routine, non-STAT, HLA typing for solid organ transplants to a single-pass NGS HLA typing method.

2 | MATERIALS AND METHODS

2.1 | Samples

Five laboratories provided a total of 289 specimens. A total of 120 specimens were provided by Baylor University Medical Center, Dallas, Texas, of which 100 comprised patients and donors having initial or confirmatory typing for solid organ transplantation (n = 57) or hematopoietic cell transplantation (n = 43). The other 20 were archived specimens with historic typing data indicative of potential novel sequences or less common alleles. Three laboratories, Baylor Scott and White Medical Center, Temple, Texas; Calgary Laboratory Services, Calgary, Alberta; and Mayo Clinic, Phoenix, Arizona, each submitted 50 specimens from kidney transplant recipients. Nineteen other specimens, comprising a local reference panel, were provided by Johns Hopkins School of Medicine, Baltimore, Maryland.

2.2 | Methods

All 289 samples had HLA typing performed at the submitting laboratory using SSOP technology (LABType, One

Lambda, Inc., Thermo Fisher Scientific Inc., Canoga Park, California) with data capture on Luminex flow cell equipment (Luminex Corporation, Austin, Texas) and analysis with Fusion software (One Lambda, Inc.), following manufacturer's instructions. Fusion software uses preset limits for positive probe signal levels but does allow operator override of individual probe reactions. Baylor University Medical Center, Calgary Laboratory Services, Mayo Clinic, and Johns Hopkins School of Medicine employed an intermediate resolution LABType SSOP system that assessed polymorphism in exons 2 and 3 for HLA-A, B, C, DQA1, DQB1, DPA1, and DPB1, while analysis of DRB1 and DRB3/B4/B5 was limited to exon 2. SSOP analysis at Baylor Scott and White Medical Center employed the LABType XR SSOP system with analysis of HLA-A and B for exons 2 to 5, HLA-C for exons 2 to 7, and DRB1 for exon 2. For typing of DQA1, DQB1, DPA1, and DPB1, Baylor Scott and White Medical Center employed the same LABType SSOP system as the other four laboratories. The SSOP typing data of the 19 specimens from Johns Hopkins School of Medicine comprised archived results of typing performed from 2008 to 2017. In addition, historic sequencing-based typing results, performed by the submitting laboratory, were available for 15 of the 20 archived specimens provided by Baylor University Medical Center and the 19 specimens from Johns Hopkins School of Medicine.

Coded DNA samples were submitted to the Fred Hutchinson Cancer Research Center (FHCRC) for HLA genotyping by NGS technology in a blinded fashion. All HLA class I and class II genotyping used ScisGo HLA v6 reagents (Scisco Genetics, Inc., Seattle, Washington) and followed manufacturer's instructions. Briefly, the method employs an amplicon-based 2-stage polymerase chain reaction (PCR) followed by pooling of samples and application to sequencing using a MiSeq v2 PE500 (Illumina, San Diego, California). Four multiplex primer sets provide complete amplification of HLA-A, B, and C at exons 1 through 7; DRB1, DRB3/B4/B5, DQA1, DQB1, and DPB1 at exons 1 through 4; and DPA1 at exons 2 through 4. For this study, NGS typing was performed in two batches using the MiSeq platform (Illumina, Inc). The first batch comprised the 120 samples from Baylor University Medical Center and was run with the MiSeq reagent kit v2, 500 cycles (Illumina, Inc.). The combined 169 samples from Mayo Clinic, Calgary Laboratory Services, Baylor Scott and White Medical Center, and Johns Hopkins School of Medicine were run in a second batch with MiSeq reagent kit v3, 500 cycles (Illumina, Inc.). Both batches used a 36-hour sequencing run time. In addition, NGS of subsets of study samples in 24 sample batches assessed reliability of 24-hour sequencing run times with MiSeq Nano kit v2 500 cycles (Illumina, Inc.). NGS data analysis employed GeMS-UI v80 software (Scisco Genetic, Inc.) according to manufacturer's instructions. The GeMS software minimal depth of coverage criteria for data acceptability is preset at 25 reads per amplicon, with flags for data review.

2.3 | DNA requirements

The SSOP technology specifies 40 ng (2 μL at 20 ng/μL) of sample DNA per PCR reaction for a total of 280 to 320 ng per sample for full HLA typing. The NGS technology accommodates 2 to 20 ng of sample DNA per PCR reaction, so that the total DNA required for complete HLA target generation may be as little as 8 ng per sample. The DNA samples provided by the submitting laboratories for the NGS analyses had a broad range of DNA concentrations and quality and represented specimens that might be encountered in any genotyping laboratory. A single set of samples was provided by the submitting laboratories, and no replacement samples were required for NGS genotyping.

2.4 | Typing concordance

Assessment of typing concordance between SSOP and NGS involved comparison of results at the level of resolution provided by SSOP. An automated data comparison program was employed by the FHCRC laboratory to query the ambiguous SSOP coded allele sets for the presence of the specific HLA allele identified by the NGS typing. Discordant results were flagged, and the NGS allele assignments were reported back to the submitting laboratory for review. NGS analysis was also reviewed in the context of the discordant results. Concordance of typing was also assessed between NGS and the historic sequence data available for 15 specimens from Baylor University Medical Center and the 19 specimens from Johns Hopkins School of Medicine.

3 | RESULTS

3.1 | Workflow comparison

Figure 1 illustrates the basic workflow of SSOP vs NGS and provides the direct technologist hands-on effort and instrument run times for a typical clinical typing run of up to 24 samples.

SSOP typing is accomplished in four basic stages. The first stage is target generation via PCR with seven to eight PCR reactions per specimen, depending on the need for HLA-B ambiguity resolution, so that up to 24 specimens are amplified in two microtiter plates. In stage 2, amplicons are transferred into two new plates for denaturation, hybridization, and labeling. In the third stage, all samples are

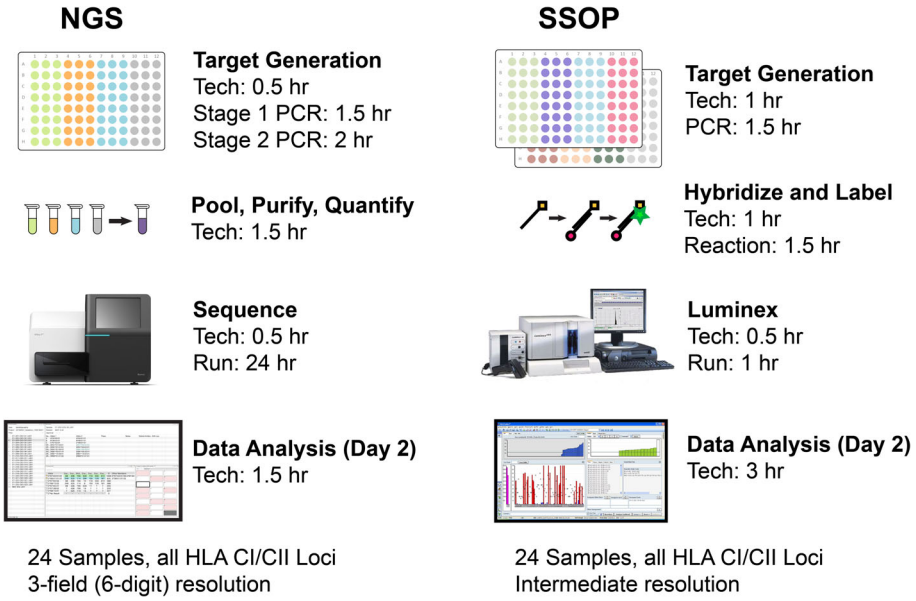

FIGURE 1 Time and effort for next generation sequencing (NGS) and sequence-specific oligonucleotide probe (SSOP) workflows. Stepwise comparisons of amplicon-based NGS and SSOP workflows, examining 24 samples for 11 HLA loci at high resolution (NGS) or at intermediate resolution (SSOP). Each workflow consists of four basic steps as indicated, with hands-on technologist times and equipment run times for the respective protocol indicated to the immediate right of each depiction. The 24-hour MiSeq run time indicated for the NGS protocol is specific for 24 sample runs using a nano-PE500 v2 Illumina kit. Larger sample numbers require the standard PE500 v2 kit which requires 36 hours to complete. These NGS data generated for this study required two 40-hour runs as >100 samples were included in each run

transferred to reading trays and run on the Luminex flow cell equipment for assessment of probe reactions. The final step involves HLA software analysis of probe patterns for genotype assignments. All hands-on wet work can be accomplished in 3 hours so that bench work for a batch of up to 24 samples, including Luminex processing, is accomplished on day 1 (~ 6 hours), with HLA software analysis, data review and reporting on day 2. In this study, SSOP typing was usually performed in batches of up to 24 specimens.

Similarly, NGS typing begins with target generation via a 1.5-hour PCR, a reagent addition, and a 2-hour extension PCR in the same plate. The multiplexed panel of four PCR reactions per specimen allows amplification of up to 24 samples in a single microtiter plate. In stage 2, amplicons from all specimens are pooled into four locus-specific tubes for purification, quantification, and pooling into a single tube. In stage 3, an aliquot of the entire sequence library is loaded on a MiSeq flow cell and a sequencing run is initiated. In the final stage, sequence data are analyzed with HLA software for allele assignments. All bench work, including initiation of the MiSeq run, is accomplished on day 1. For a typical clinical batch of up to 24 samples, hand-on technologist effort is approximately 3 hours from PCR setup through instrument loading. The 24-hour sequencing run, on the MiSeq Nano kit v2 500 cycles, provides results for reporting on day 2. For larger sample batches, such as the 120 and 169 batches run in this study, all bench work and instrument loading are accomplished in under 8 hours on day 1, followed by a 40-hour sequencing run, with results available on day 3.

3.2 | NGS metrics

With few exceptions, all NGS allele assignments of the 120 and 169 sample batches passed the GeMS preset minimal 25 reads per amplicon depth of coverage criteria for data acceptability. In one case, a DRB4 allele exhibited low exon 1 reads because of a novel polymorphism in a 5′ primer as described in more detail below in the novel allele Section 3.5. In another case, no reads for exons 2, 3, and 4 were detected in conjunction with a DRB5 exon 1 sequence with an acceptable read level, also described in the novel allele section. The blinded results of these two large NGS batches were the allele assignments used for evaluation of concordance with SSOP typing. The extended NGS testing of 24 sample batches consistently provided acceptable data coverage and accurate typing results using the 24-hour MiSeq Nano kit.

3.3 | Concordance of typing results

At the level of resolution provided by SSOP, the HLA-A, B, and C typing data from NGS and SSOP were fully concordant. At HLA class II, DRB1, DRB3, DRB4, DRB5, DQA1,

TABLE 1 Discordant typing results

NGS typing	SSOP typing			
*DPB1*23:01:01, 81:01*	***DPB1*02:01, 04:01***	1 probe false negative		
*DRB5*01:01:01, 02new*[a]	***DRB5*01:02/01:08 N, 01:06***	2 probes false negative[b]		
*DRB1*04:10:01, 14:02:01*	***DRB1*04:XX, 14:XX***	1 probe misassigned	***DRB1*04:XX* excluded **04:10***	***DRB1*14:XX* excluded **14:02***
*DRB1*04:04:01, 08:11*	DRB1*04:XX, ***08:XX***	1 probe misassigned	***DRB1*08:XX* excluded **08:11***	DRB1*04:XX included **04:04*
*DQB1*03:19:01, 05:01:01*	*DQB1**03:XX**,** 05:XX	1 probe false negative	***DQB1*03:XX* excluded **03:19***	*DQB1*05:XX* included **05:01*
*DQA1*01:01:02, 04:01:01*	*DQA1*01:SXYS*, ***06:01***	1 probe false negative	DQA1*01:SXYS included **01:01*	
*DQB1*06:09:01, 06:49*	DQB1****06:XX1***, 06:XX2	1 probe misassigned	***DQB1*06:XX1* excluded **06:49***	*DQB1*06:XX2* included **06:09*
*DQB1*03:02:01, 06:04:01*	DQB1****02:01***, 06:XX1	3 probes false negative[c]	*DQB1*06:XX1* included **06:04*	

Note: Bold, italic font indicates a discordant, incorrect SSOP allele assignment.

Abbreviations: NGS, next generation sequencing; SSOP, sequence-specific oligonucleotide probe; SSP, sequence-specific primer.

[a]*DRB5*02new* with an exon 4 SNP.

[b]SSP by submitting lab concordant with NGS.

[c]Repeat SSOP by submitting lab concordant with NGS.

DQB1, DPA1, and DPB1 results were concordant except for 11 allele assignments among eight specimens (Table 1). Three specimens had discordant typing for both alleles at a locus. In one, NGS assigned *DPB1*23:01:01*, *81:01* while SSOP typing showed *DPB1*02:01, 04:01*. Review of DPB1 SSOP probe reactions identified one probe reaction slightly below the standard cutoff for a positive reaction, which, if positive, gave typing concordant with NGS. For another specimen, NGS assigned *DRB5*01:01:01, 02* new (exon 4 single nucleotide polymorphism [SNP]) vs SSOP typing of *DRB5*01:02/01:08 N, 01:06*. SSP analysis by the submitting laboratory was concordant with the NGS allele assignments. Review of DRB5 probe reactions revealed two false-negative probe reactions. One other specimen was typed by NGS as *DRB1*04:10:01, 14:02:01*, while the SSOP coded allele sets excluded those alleles. Review found that one probe reaction had been changed or "misassigned." In another four specimens, a single allele was discordant between NGS and SSOP: a DQA1 allele with one false-negative probe; a DQB1 allele with one false-negative probe; *a DRB1*08* allele with a "misassigned" probe; and a *DQB1*06* allele with a "misassigned" probe. One other discordant result involved SSOP typing as *DQB1*02:01*, *06* vs *NGS of DQB1*03:02:01*, 06:04:01. Review found three false-negative probes, and repeat SSOP was concordant with NGS.

NGS results were concordant with the historic sequencing results provided for the 19 specimens from John Hopkins School of Medicine and 15 specimens from Baylor University Medical Center, including novel sequences in three samples.

TABLE 2 Ambiguity resolution NGS vs SSOP

			Percent resolution to specific alleles				
Laboratory	**Typing technology**	**Samples (n)**	**HLA-A (%)**	**HLA-B (%)**	**HLA-C (%)**	**DRB1 (%)**	**Mean % allele resolution (n)**
Baylor Scott and White Medical Center	HR SSOP	50	20	25	5	19	17 (69)
Mayo Clinic	IR SSOP	50	0	1	0	2	0.75 (3)
Calgary Lab Services	IR SSOP	50	0	0	0	2	0.5 (2)
Baylor University Medical Center	IR SSOP	120	1.7	2	0.8	1.7	1.5 (19)
Johns Hopkins School of Medicine	IR SSOP	19	0	5.3	0	0	1.3 (2)
Fred Hutchinson Cancer Research Center	NGS	289	99.3	99.5	100	100	99.7 (2312)

Abbreviations: HR SSOP, high-resolution XR LABType; IR SSOP, Intermediate resolution LABType.

3.4 | Level of resolution

For most specimens, SSOP generated ambiguous results at all loci with codes employed to represent sets of the possible specific alleles. Table 2 summarizes the percent of instances, in which a single-specific allele was assigned by SSOP across the highly polymorphic HLA-A, B, C, and DRB1 loci. With the intermediate resolution SSOP employed by 4 laboratories, less than 2% of results included a specific allele assignment. Even with the extended higher-resolution SSOP system employed at the Baylor Scott and White Medical Center, only 17% of assignments were specific alleles across the four loci. For the less polymorphic DP and DQ loci, with intermediate resolution SSOP by all five laboratories, specific allele assignments ranged from a low of 1.5% for DQB1, to 11.8% for DPB1, 26.6% for DQA1, and a high of 37.9% at DPA1. Similarly, most SSOP results for DRB3, DRB4, and DRB5 were ambiguous. Figure 2 illustrates the complex typing results for a heterozygous specimen generated by the intermediate resolution SSOP system. In the example, all SSOP assignments are ambiguous except for one DPA1 allele. *DPA1*01:03* was specifically assigned as the SSOP kit included a probe that queried position 125 C, which is present in *DPA1*01:03*, but in combination with an ambiguous *DPA1*01:CU*. The CU code indicates possible *DPA1*01:03* or *DPA1*01:13*. The latter allele cannot be ruled out because the probe panel does not interrogate the position 125 T of *DPA1*01:13*. With the extended higher-resolution SSOP system for HLA-A, B, C, and DRB1, the level of resolution depended heavily on the specific typing of a sample with a few alleles specifically identified in any

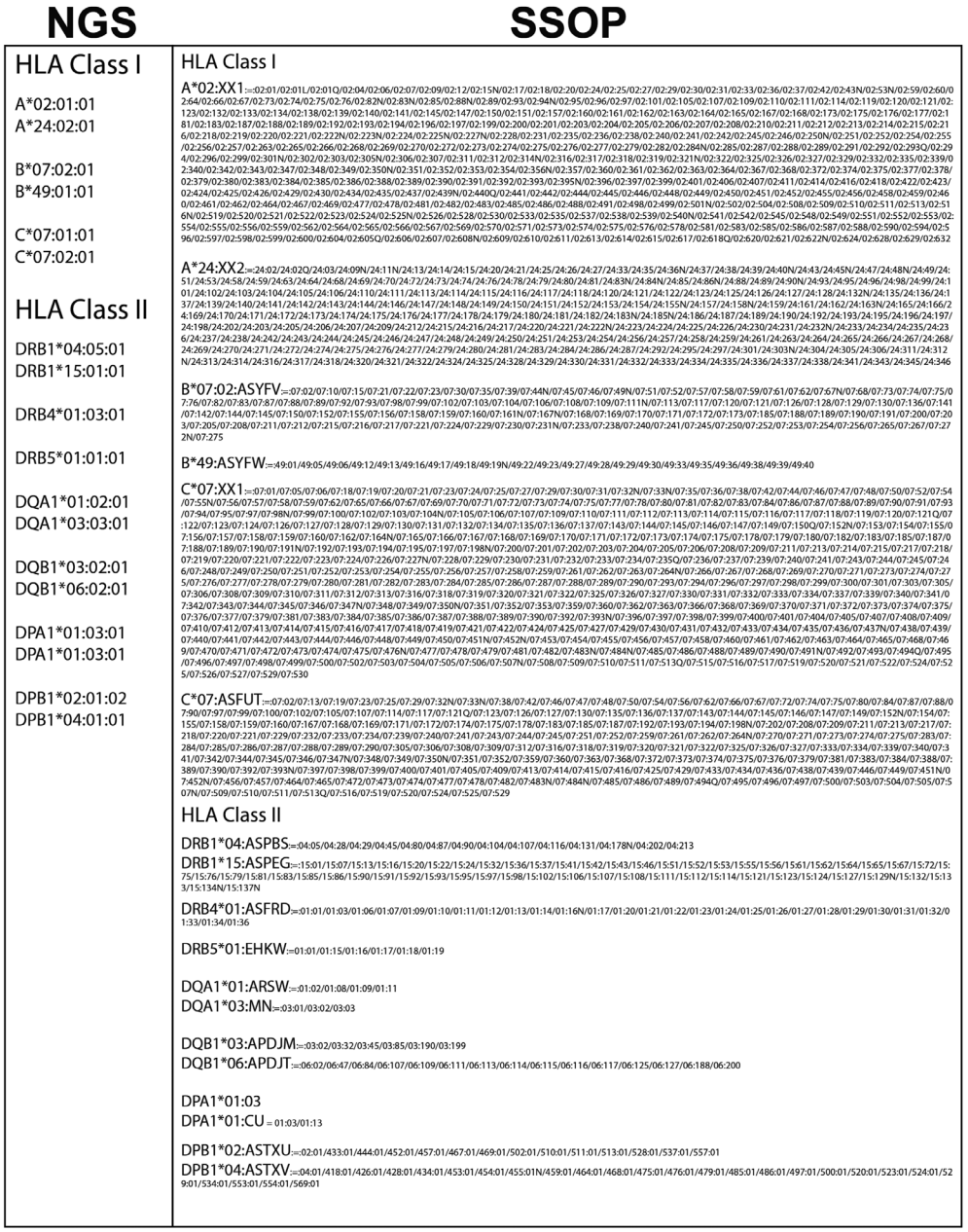

FIGURE 2 Allelic ambiguities of next generation sequencing (NGS) vs sequence-specific oligonucleotide probe (SSOP). A typical example of HLA typing for the same sample illustrates the different levels of resolution achieved by NGS and SSOP. Amplicon-based NGS typing yielded three-field types for all loci (no ambiguities at the three-field level), while intermediate-resolution SSOP typing gave two-field types with varying levels of ambiguity. SSOP for HLA class I shows the highest ambiguity levels ranging from *HLA-A*02:XX1* and *C*07:XX1* each with over 300 ambiguous allele calls down to *HLA-B*49:ASYFW* with 21 ambiguous variants (small print after each coded call in large text)

heterozygous combination. Resolution ranged from no specific alleles assigned in 15 specimens, up to five specific allele assignments across HLA-A, B, C, and DRB1 seen in three specimens.

With very few exceptions, NGS provided unambiguous specific HLA allele assignments at the three-field level, within the limits provided by analysis of class I exons 1 to 7 and class II exons 1 to 4. The few NGS typing ambiguities observed were specific discrete diploid allele combinations. Among the highly polymorphic HLA-A, B, C, and DRB1 loci (Table 2), four HLA-A alleles in two samples and three HLA-B alleles in two other samples had ambiguous NGS assignments. All NGS results for HLA-C and DRB1 were unambiguous specific alleles. At the less polymorphic loci, NGS provided specific alleles for all samples at DQA1, DPA1, DRB3, and DRB5, while 97.7% of DQB1 alleles were specific, and 68.5% of DPB1 assignments were specific alleles. Table 3 lists the specific NGS ambiguous diploid combinations observed in this study, with one at HLA-A, two at HLA-B, two at DQB1, and 13 ambiguous diploid combinations at DPB1. In addition, a specimen with two *DRB4* genes may have a diploid ambiguity because of the unusual *DRB4*03:01N*[14] allele, which encodes exons 3 and 4 identical to *DRB4*01:01:01* but has no exon 1 or exon 2 sequences. Thus, *DRB4*01:01:01 and DRB4*03:01N* cannot be discriminated in the presence of another *DRB4*01:01:01* or *DRB4*01:03:01:01* or *DRB4*01:03:02.*

3.5 | New alleles identified by NGS

NGS analysis identified 21 novel sequences (Table 4), with six novel DPA1 alleles, three each of HLA-C, DRB1, and DRB3, two each of DQA1 and DRB5, and one novel sequence at each of DRB4 and DPB1. Seven of these samples were from the archived panel from Baylor University, assembled for their potential uniqueness among samples processed there. However, all the samples with novel alleles were clinical samples, including the archived specimens. SNPs were responsible for 20 of the novel sequences, while one appeared to be the result of a multiexon deletion. Thirteen of the novel alleles carried SNPs in exon 4, while three alleles had SNPs in exon 3, two in exon 2, one each in exon

TABLE 3 NGS diploid ambiguities observed among 289 specimens[a,b]

Allele 1	Allele 2	vs	Allele 3	Allele 4
*A*02:01:01*	**68:01:02*		*A*02:614*	*A*68:164*[c]
*B*15:01:01*	*B*44:02:01*		*B*15:247*	*B*44:27:01*[c]
*B*15:01:01*	*B*44:216*		*B*15:247*	*B*44:216*[c]
*DPB1*04:01:01*	*DPB1*04:02:01*		*DPB1*105:01*	*DPB1*126:01*[d]
*DPB1*03:01:01*	*DPB1*04:01:01*		*DPB1*124:01*	*DPB1*350:01*[d]
*DPB1*02:01:02*	*DPB1*04:02:01*		*DPB1*105:01*	*DPB1*416:01*[d]
*DPB1*01:01:01*	*DPB1*02:01:02*		*DPB1*162:01*	*DPB1*461:01*[d]
*DPB1*04:02:01*	*DPB1*17:01:01*		*DPB1*105:01*	*DPB1*460:01*[d]
*DPB1*03:01:01*	*DPB1*04:02:01*		*DPB1*351:01*	*DPB1*463:01*[d]
*DPB1*04:01:01*	*DPB1*13:01:01*		*DPB1*133:01*	*DPB1*350:01*[d]
*DPB1*03:01:01*	*DPB1*05:01:01*		*DPB1*104:01:01*	*DPB1*135:01*[d]
*DPB1*03:01:01*	*DPB1*519:01*		*DPB1*104:01:01*	*DPB1*13:01:01*[d]
*DPB1*04:01:01*	*DPB1*14:01:01*		*DPB1*350:01*	*DPB1*651:01*[d]
*DPB1*03:01:01*	*DPB1*105:01*		*DPB1*124:01*	*DPB1*463:01*[d]
*DPB1*01:01:01*	*DPB1*17:01:01*		*DPB1*131:01*	*DPB1*162:01*[d]
*DPB1*04:02:01*	*DPB1*23:01:01*		*DPB1*105:01*	*DPB1*138:01*[d]
*DQB1*06:02:01*	*DQB1*06:04:01*		*DQB1*06:39*	*DQB1*06:84*[d]
*DQB1*06:02:01*	*DQB1*06:09:01*		*DQB1*06:84*	*DQB1*06:88*[d]
*DRB4*01:01:01*	*DRB4*01:01:01*		*DRB4*01:01:01*	*DRB4*03:01N*[e]
*DRB4*01:01:01*	*DRB4*01:03:01:01*		*DRB4*03:01N*	*DRB4*01:03:01:01*[e]
*DRB4*01:01:01*	*DRB4*01:03:02*		*DRB4*03:01N*	*DRB4*01:03:02*[e]

Abbreviations: NGS, next generation sequencing; SSOP, sequence-specific oligonucleotide probe.

[a]All NGS ambiguities are also present within SSOP typing results.

[b]No NGS diploid ambiguities observed at HLA-C, DRB1, DRB3, DRB5, DQA1, and DPA1.

[c]Ambiguous phasing of exons 3 and 4.

[d]Ambiguous phasing of exons 2 and 3.

[e]Ambiguity due to unique structure of *DRB4*03:01N*.

TABLE 4 Novel sequences identified by NGS[a]

Likely common parent allele	SNP exon location	Nucleotide location	SNP	Codon location	Codon change	Amino acid change	Protein change	Reported in IMGT 2018[b]
*C*01:02:01*[c]	3	419	A > T	116	TAC > TTC	Y > F	Yes	*C*01:146:02*
*C*04:01:01*[c]	4_5	855_979	T > G_A > G	261_303	GTT > GTG_ATG > GTG	V > V_M > V	No_Yes	*C*04:339:02*
*C*07:18*[c]	4	895	G > A	275	GAG>AAG	E > K	Yes	*C*07:621*
*DPA1*01:03:01*	4	714	C > T	207	ATC > ATT	I > I	No	
*DPA1*01:03:01*	2	138	G > A	15	ACG > ACA	T > T	No	
*DPA1*01:03:01*	4	705	C > G	204	GTC > GTG	V > V	No	
*DPA1*01:03:01*	4	763	C > T	224	CGG > TGG	R > W	Yes	
*DPA1*02:07*[d]	4	764	G > A	224	CGG > CAG	R > Q	Yes	*DPA1*02:12*
*DPA1*03:01*[c]	4	705	G > C	204	GTG > GTC	V > V	No	*DPA1*03:01:02*
*DPB1*04:01:01*	4	668	G > A	194	CGG > CAG	R > Q	Yes	*DPB1*849:01*
*DQA1*04:01:01*	2	309	C > T	80	TCC > TCT	S > S	No	
*DQA1*01:01:01*	4	638	C > T	190	TCA > TTA	S > L	Yes	
*DRB1*04:04:01*[e]	4	746	A > G	220	TAC > TGC	Y > C	Yes	
*DRB1*03:01:01*[c]	4	714	C > T	209	GGC > GGT	G > G	No	
*DRB5*02:02*[c,f]	4	694	A > G	203	ATC > GTC	I > V	Yes	*DRB5*02:21*
*DRB5*02:02:01*	3	445	A > G	120	AAT > GAT	N > D	Yes	
*DRB3*01:01:02*[g]	1	74	G > C	−5	CGA > CCA	R > P	Yes	
*DRB3*02:02:01*	4	670	G > T	195	GCA > TCA	A > S	Yes	
*DRB3*02:02:01*	3	490	G > A	135	GGC > AGC	G > S	Yes	*DRB3*02:86*
*DRB4*01:03:01*	SNP 90 bp upstream of exon 1.						No	
*DRB1*15:01:01*[c,h]	Deletion mutation						Yes	

Abbreviations: NGS, next generation sequencing; SNP, single nucleotide polymorphism.

[a]Except as noted below, a single example of each allele was observed.

[b]IPD-IMGT/HLA database. Release 3.35.0, January 23, 2019.

[c]Submitted by Baylor Transplant Laboratory in archived specimen panel.

[d]Found in two unrelated individuals.

[e]Two siblings.

[f]Found in two unrelated individuals.

[g]Found in four unrelated individuals.

[h]Exon one sequence of *DRB1*15* is present. *No DRB1*15* sequence detected for exons 2, 3.

1 and exon 5, and one (*DRB4*01new*) in the 5′ untranslated region upstream of exon 1. One of the novel *HLA-C*04* alleles carried two SNPs, one in exon 4 and one in exon 5. Most of the SNPs (n = 13) resulted in amino acid substitutions, while eight SNPs were silent substitutions with no peptide change. Over the past year, because the NGS analysis was performed in early 2018, eight of these 21 novel sequences have been reported and given allele names in the IMGT/HLA database[15] as indicated in Table 4.

Four novel sequences were identified in two or more samples, all with coding polymorphisms. Two siblings carried *DRB1*04new* with an exon 4 SNP and shared the haplotype *A*33:01:01~C*08:02:01~B*14:02:01~DRB1*04new~DRB4*01:03:03~DQA1*03:01:01~DQB1*03:02:01~DPA1*01:03:01~DPB1*03:01:01*. The *DRB5*02new* allele, with an exon 4 A > G SNP, was found in two unrelated specimens, both of which carried *DRB1*16:02:01~DQA1*01:02:02~DQB1*05:02:01*. Similarly, the two unrelated specimens with *DPA1*02new* carried a coding SNP in exon 4, *and both carried DPB1*85:01*. The novel DRB3 sequence was found in four apparently unrelated individuals submitted from three different laboratories. In two cases, the exon 1 codon −5 arginine (CGA) to proline (CCA) was clearly encoded on a *DRB3*01* allele. The other two specimens carried two DRB3 alleles and, although NGS analysis did not identify which allele was novel, it is likely that all four examples have the same novel *DRB3*01* allele. All four specimens with this novel *DRB3* sequence carried *DRB1*03:02:01*, *DQA1*04:01:01*, and *DQB1*04:02:01*. Sixteen other novel sequences with SNPs were detected in single individuals, with sequence details shown in Table 4.

The novel sequence with an apparent multiexon deletion mutation was identified in one specimen with SSOP typing of *DRB1*14* only, *DRB3*02*, *DRB5*01*, and *DQB1*05, 06*. Sanger SBT by the submitting laboratory had confirmed the presence of *DRB1*14:54*, *DRB3*02:02*, *DRB5*01:01*, and *DQB1*05:03*, *06:02* and the absence of a *DRB1*15* allele sequence. NGS analysis identified *DRB1*14:54:01*, *DRB3*02:02:02*, *DRB5*01:01:01*, and a *DRB1*15* exon 1 sequence, but without any evidence of *DRB1*15* sequences for exons 2 and 3. Because the exon 4 sequences for *DRB1*14:54:01* and *DRB1*15* are identical, the presence or absence of an exon 4 sequence for the *DRB1*15* allele could not be determined.

4 | DISCUSSION

4.1 | Workflow efficiency and turnaround time

In a routine clinical setting for typing of up to 24 specimens, both SSOP and NGS protocols facilitate a 2-day TAT. However, the SSOP bench work complexity, with two full plate transfers of all samples, multiple reagent additions, and several plate centrifugation cycles, mitigates against scale-up over 24 specimen batches. Depending on the laboratory requirement for resolving and reporting ambiguous SSOP data, the time for analysis and result reporting may be extended. Also, while infrequent, SSOP typing errors involved false-negative probe reactions in five samples and "misassigned" probes in three samples, because of a reviewer-introduced change in a probe reaction to assign more common alleles or combinations of more common/expected alleles. When detected, questionable SSOP typing results may require additional testing using different technology, leading to an increase in TAT because of the time necessary to perform the extra testing. Even beyond, the direct labor and material costs of extended testing are the increased expenses for maintaining proficiency in multiple discrete technologies.

The amplicon NGS technology employed in this study allows initial target generation of 24 samples in a single microtiter plate. After initial PCR, one reagent is added for the extension PCR in the same plate, followed by amplicon pooling. This efficient workflow, with far fewer sample manipulations, minimizes assay and technologist variability. Although not explicitly demonstrated within this study, the amplicon-PCR system also provides reliable typing with DNA isolated from buccal swabs and blood spots. The extensibility of NGS to accommodate high-volume typing gives flexibility for scale-up of the laboratory typing capability. Thus, compared with SSOP, routine NGS typing reduces costs of consumables and hands-on technologist time, while maintaining a 2-day TAT for reporting and, as shown here, providing a vast improvement in the level of resolution.

4.2 | Accuracy and level of resolution

The high concordance of NGS and SSOP (>99%) attests to the accuracy of both HLA typing systems. Ambiguities in SSOP analysis result from lack of coverage for entire exons and a lack of probes for all polymorphisms within the exons analyzed. In addition, diploid ambiguities are generated by the inability to determine the phase of polymorphism/s in heterozygous specimens, either within an exon or across exons. With few exceptions, the intermediate resolution SSOP results exhibited ambiguous typing in all samples at all loci. Both coverage and phasing, often operating together, contributed to the high frequency and the complexity of ambiguous SSOP data. With the higher-resolution SSOP, certain alleles are specifically assigned in any heterozygous combination. However, even with the higher-resolution SSOP, only 17% of HLA-A, B, C, and DRB1 results were specific allele assignments, and the continual discovery of novel HLA alleles would inevitably be

expected to increase the number and complexity of ambiguous typing results generated by the SSOP technology.

The NGS technology covered exons not assessed by SSOP at all loci, provided full-length exon sequence data, as well as certain intronic sequences that included polymorphisms associated with the known HLA null alleles. With few exceptions, NGS eliminated ambiguous heterozygous combinations through the sequencing of individual DNA molecules and through overlapping sequences that spin and link certain exons. The HLA-A, B, DQB1, and DPB1 diploid ambiguities occurred because the introns between exons 2 and 3 of DPB1 and DQB1 or between exons 3 and 4 of HLA-A and HLA-B were not completely sequenced. Supplemental reagents for sequencing across those regions are available but would add to costs and extend TAT for reporting. Most of the diploid ambiguities involved combinations of two common alleles vs two infrequent or rare alleles. It should be noted that the few ambiguities encountered with NGS were also present within the SSOP typing results.

The *DRB4*01new* allele with a SNP within a primer site illustrates a strength of the amplicon-based NGS technology employed in this study compared with NGS based on long-range PCR. Although exon 1 amplification was weak or absent, amplification and sequencing of the DRB4 allele for exons 2 through 4 was normal and clearly indicated the presence of a DRB4 gene. In long-range PCR, a primer site polymorphism could negate amplification of the full locus and generate a hemizygous type leading to an erroneous homozygous call at that locus. By design, the independent amplification of multiple segments of one gene makes the amplicon NGS system less subject to false homozygosity. Conversely, an advantage of the long-range approach is the ability to phase between some exons relative to the amplicon approach, with the most common occurrence of this in HLA-DPB1 (Table 3). In many of these cases, a long-range shotgun approach will phase exons while the amplicon approach, which depends on database lookup for phase, will encounter a diploid ambiguity. This limitation can be weighed against the relative simplicity in laboratory execution of the amplicon approach used in this study, with overall parallels much more in line with the SSOP protocol in that regard.

5 | ALTERNATIVE NGS HLA TYPING APPROACHES

A number of major transitions in technology platforms utilized for HLA typing have taken place over past decades, and the emergence of NGS for HLA surely marks the latest of these transitions. HLA typing for hematopoietic cell transplants has already seen the introduction of NGS HLA typing in clinical laboratories, and with careful attention to key metrics, provides an effective replacement of other technologies.[16-18] In this context, a continuation of new alleles is discovered, in particular with respect to the ability of NGS technologies to examine most or all exon sequences over the subset of exons examined using older methods.[18] While currently in limited use in clinical typing laboratories, these methods have been and continue to be very effectively used in establishing more comprehensive database entries for registries.[19-21] Interestingly, while the long-range shotgun methods do examine intronic sequences, a relatively low frequency of novel intronic variation has been found,[21,22] and no functional consequences have been established for the occasional new variant that has been described. The current study was undertaken to examine the effectiveness of a novel amplicon-based NGS approach to improve on typing methodology for solid organ transplants, a clinical arena where SSOP HLA methods predominate. The main improvement over other NGS approaches in this context is the horizontal translation of the amplicon approach with respect to the variables discussed above—perhaps most notably workflow simplicity and cost—while providing essentially the same levels of accuracy and consequential allelic resolution as other NGS approaches.

6 | CONCLUSIONS

Ultimately, the impetus for moving to a new technology lies in the implications for patient care. Duquesnoy et al[4] presented a strong argument for high-resolution typing of patient and donor for highly sensitized patients, particularly in the setting of living donor transplants. Routine use of high-resolution NGS typing for solid organ transplant candidates potentially provides valuable information, including identification of rare alleles that can have a devastating impact on graft survival.[23] When possible, use of NGS for confirmatory typing of the donor organ may better define the level of matching and facilitate posttransplant monitoring for the development of de novo donor-specific antibodies. While the 2-day NGS TAT is not suitable for initial deceased donor workups in solid organ transplantation, the exquisite sensitivity and specificity of NGS genotyping, with virtually complete ambiguity resolution, is a strong impetus for laboratories to consider implementing single-pass NGS for routine HLA typing in support of solid organ transplantation.

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CONFLICT OF INTEREST

A.G.S. is a scientific consultant in Scisco Genetics, Inc., Seattle, Washington. S.P. and C.W. P. are scientific advisors in Scisco Genetics, Inc. W.C.N. is an employee in Scisco Genetics, Inc. Medhat Askar: Scientific Advisory Board, Illumina, Inc., San Diego, California. D.E.G. is the president and CEO in Scisco Genetics, Inc. A.J., S.T.S., F.M.K., N.B., A.A.M., M.J.P., C.Y.U., M.P.B., A.W. declare no potential conflict of interest.

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