Human Immunology 77 (2016) 820–823

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Invited Review

Clinical validation of NGS technology for HLA: An early adopter's perspective

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ARTICLE INFO

Article history:
Received 25 April 2016
Revised 26 May 2016
Accepted 20 June 2016
Available online 21 June 2016

Keywords:
NGS
HLA alleles
Validation
Hematopoietic stem cell transplantation
Solid organ transplantation

ABSTRACT

Clinical validation of NGS for HLA typing has been a topic of interest with many laboratories investigating the merits. NGS has proven effective at reducing ambiguities and costs while providing more detailed information on HLA genes not previously sequenced. The ability of NGS to multiplex many patients within a single run presents unique challenges and sequencing new regions of HLA genes requires application of our knowledge of genetics to accurately determine HLA typing. This review represents my laboratory's experience in validation of NGS for HLA typing. It describes the obstacles faced with validation of NGS and is broken down into pre-analytic, analytic, and post-analytic challenges. Each section includes solutions to address them.

1. Introduction

Sanger sequencing has been the gold-standard for determining high-resolution human leukocyte antigen (HLA) typing for many years [1,2]. While Sanger sequencing is still a viable option for some clinical laboratories, primarily low-volume centers, many clinical HLA laboratories are switching to next-generation sequencing (NGS) technologies. NGS is best described as single molecule sequencing and is a high-throughput methodology that allows multiple patient samples to be run concurrently. NGS allows for deeper interrogation of the genomic sequences. This is particularly important for the HLA genes because they have the highest degree of polymorphism within the human genome [3,4]. Compared to Sanger sequencing, NGS has a significantly lower ambiguity rate, higher-throughput, and lower costs for multigenic disorders [5–9]. However, there are disadvantages to NGS. These include potentially longer turnaround times (TAT), additional instrumentation costs, and sequencing of HLA genes not yet clinically relevant. Fortunately for HLA laboratories, NGS has been utilized in clinical molecular genetics laboratories for many years and that experience can guide HLA laboratories considering NGS. This article describes the best practices, challenges, and lessons our laboratory learned through our validation of NGS for HLA typing for the American Society of Histocompatibility and Immunogenetics (ASHI).

The ASHI guidelines for validation of NGS for HLA typing went through several iterations before, during, and after the completion of our validation. On December 17, 2015, the Accreditation Review Board (ARB) and the ASHI Board of Directors approved a revised version of the guidelines based on input by experienced early adopters. The updated guidelines returned much of the decision-making to the Laboratory Director, but also clarified several points for laboratories considering NGS. Of note is the guidance to included appropriate criteria for acceptance of sequencing runs and a policy for how a laboratory will handle the new influx of data, particularly novel alleles.

2. Materials and methods

Our laboratory uses a commercially available assay sold by Illumina (version 1) on the MiSeq platform. We have recently completed our validation of the second version of Ilumina's assay and will be implementing it in May 2016. The second version has several significant improvements over version 1, including sample pooling (already done by Omixon) and improved ambiguity resolution. The two-field ambiguity rate for TruSight HLA version 1 is 3.5% [10] and <0.5% for version 2 (unpublished data). The ambiguity rate for Holotype is <0.8% [11]. All of our NGS libraries are prepared manually by 1–2 technologists. HLA genotypes were called

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http://dx.doi.org/10.1016/j.humimm.2016.06.014
0198-8859/

using the vendor provided software Assign for TruSight HLA using the best matching allele. We have evaluated assays from GenDx (NGSgo) and Omixon (Holotype) and have not evaluated OneLambda's (NXType) or Immucor (MIA FORA) assays. Each assay has strengths and weaknesses a laboratory should consider prior to implementation. Overall, there are minor differences in sequenced regions, coverage consistency, and data quality from each assay [11–13]. All of the vendor assays utilize long-range PCR of the HLA genes for amplification with many genes being fully sequenced. The decision of which vendor to use is dependent on a number of factors, such as instrument availability, cost, and throughput relative to your laboratory's workload.

Our decision to use Illumina's HLA assay was based on availability of DPB1 typing at the time of validation (currently available from all vendors), software included with assay purchase (Omixon includes software, GenDx does not), and no additional instrumentation required (MiSeq already on-site).

3. Results

The guidelines for proper validation of any clinical assay are laid out in CLIA 88′ (42 CFR 493). That regulation states assay validations should include determination of acceptable specimen types, accuracy, precision (inter- and intra-assay), quality assurance (QA), and quality control (QC) measures. Validation of HLA typing by NGS technologies must not only adhere to the aforementioned criteria, but should also contain an analysis of expected HLA types based on your patient population. However, it is less clear how many samples clinical HLA laboratories should analyze and what specific sequencing data should be used for QA and QC purposes.

Our validation consisted of 19 NGS runs and >210 samples (including 57 blinded samples). All libraries were prepared manually and the overall time from isolation of genomic DNA to reporting of HLA typing results was approximately 68 h. The validation took 9 months to complete. A database was constructed with detailed information on every NGS run to determine which metrics would best define acceptable runs. The metrics chosen were derived from validation samples where allelic dropout, mistypings or inadvertent typings, sample quality issues, technical errors, or manufacturer issues occurred. While those occurrences were rare (<5% of cases) with all of the available kits, those samples taught us much about the inherent issues with the assay [10]. Our experience suggests 100 samples are sufficient to assess the quality of HLA typing from various technologies based on the frequency we observed of allelic dropout, technical errors, and manufacturer issues. HLA typing 100 samples at HLA-A, -B, -C, -DRB1, -DQB1, -DPB1 with an anticipated allelic dropout rate of 0.6% (13/1938) of loci tested, would enable detection of up to 4 cases of allelic dropout. In addition, we ran over 60 samples before we had our first case of allelic dropout from a buccal swab. The samples chosen for the validation consisted of past ASHI proficiency testing (PT) samples sequenced by Sanger sequencing. Two-field resolution is sufficient for clinical HLA typing, so this was set as the gold-standard for typing. We initially applied for ASHI accreditation with 79 samples (including 10 blinded specimens). The accuracy was the same between the initial (99.9%) and final submissions (99.9%). The few inaccurate HLA typed samples were found to have been related to software glitches (subsequently fixed) or manual error. The overall ambiguity rate was found to be 3.5%, compared to 53% for Sanger sequencing. Seventy-four percent of the ambiguities were within the DRB1 and DRB4 loci, which have been subsequently addressed in the updated assay (TruSight HLA v2) [10]. Although unlikely to occur, we experienced instrument malfunction (debris within system) and loss of instrument focus with the extra samples, issues that won't typically be seen with only a few NGS runs. However, we believe those experiences highlighted the benefit of utilizing a sequencing control material on every run. The blinded samples were from the Pilot study from the 17th International HLA and Immunogenetics Workshop (IHIWS) and the standard was consensus typing from 6 laboratories using NGS-based HLA typing from various NGS vendors. A minimum of 2 blinded NGS runs using your laboratory's sample volume should be sufficient to assess laboratory policies and practices for NGS with at least 80% concordance (from the ASHI NGS Guidance) with the two-field HLA typing.

4. Discussion

Validation of NGS for HLA typing has many challenges for clinical laboratories. Those challenges are broken down into pre-analytic, analytic, and post-analytic categories.

4.1. Pre-analytic challenges

The most prominent pre-analytic challenges we experienced were HLA typing from low concentration buccal swabs and rotating the known HLA typed sample to QC all the indices used in library preparation. Most HLA laboratories are familiar with the issues presented by isolation of genomic DNA from buccal swabs; however, these issues become more pronounced when the downstream application is NGS. The shorter DNA fragment sizes often observed from buccal swabs prevent successful long-range PCR amplification and can lead to allele dropout. Fragmented DNA impacted allele dropout more than improper magnetic bead handling, technical error, or low DNA concentration. DNA being used for NGS should be quantified using a fluorometric dsDNA assay (QuBit, PicoGreen, etc.). UNC uses QuBit (ThermoFisher) for DNA quantification prior to long-range PCR. We have successfully HLA typed samples with a concentration of 2 ng/μL and will attempt NGS on low concentration samples on a case-by-case basis. However, DNA samples with low (less than 10 ng/μL) concentration can lead to allele dropout. Cases of allele dropout can be identified by comparing HLA typing results to known HLA associations, use of HLA haplotype recognition software [14], or use of automated liquid handlers to reduce the number of technical errors.

Incorporated in the updated ASHI standards (D.5.2.11.4) is the requirement for internal control samples and/or vendor supplied quality control material. For Illumina sequencers there is a control material available, PhiX, from which the sequencer will automatically determine an error rate based on the reference genome. PhiX is a small bacteriophage with a known genome that acts as a control for the MiSeq sequencing independent of NGS application (HLA, cancer genomics, RNA-Seq, etc.). Oxford Nanopore also provides similar reference material. Currently, ThermoFisher and Pacific Biosciences systems do not offer reference material for their respective sequencing systems. With these systems, a laboratory must use their own internal control sample. Importantly, National Institute of Standards and Technology (NIST) provides RM 8398, which is extracted DNA with known sequencing mutations that can also be used as a known sample regardless of NGS platform. Another ASHI standard (D.5.2.11.2) is to ensure fidelity of the indices utilized for multiplexing. This is easily done by rotation of a previously HLA typed sample. By utilizing vendor supplied material and previous HLA typed samples, laboratories can meet both ASHI standards and have an effective QA and QC monitor for NGS performance.

Our typical MiSeq runs average an error rate of 1.2%, which we monitor for instrument QA [10]. An error rate of 1.2% refers to the error rate of the system and is related to the quality of the data being generated, but not specifically related to HLA genotype. As

shown in Table 1, monitoring parameters such as error rate and cluster density can provide effective QA and QC. For example, with one NGS run the error rate increased to 2.35% due to a bubble in the system and, with another run, the flow cell was overclustered (1688 K/m^2). Many of our samples had poor quality data with both of these runs. Overclustering is an issue specific to Illumina sequencers and can easily be identified by comparing clusters generated to clusters passing the filter within Illumina's sequence analysis viewer software. Normally the two numbers are very close; however, when overclustering occurs, the amount of clusters passing the filter decreases. Overclustering prevents accurate determination of individual basepairs because the clusters of single molecules are overlapping. This prevents the Illumina sequencer from accurately identifying which basepair was incorporated. The impact of overclustering is reduced overall data quality regardless of application. If you experience overclustering, there is likely enough high quality data to provide accurate HLA genotyping; however, utilizing a known HLA typed sample can aid in that determination. These incidences were noticed prior to data analysis, which allowed for expedited troubleshooting and prevented unnecessary delay in repeat testing.

Laboratories performing NGS for HLA typing should consider where each sample is located on a sequencing plate. Samples that are likely to have similar typings (i.e. NMDP samples and siblings) should be placed distant from each other during library preparation. Although unlikely to occur, increased spacing between potentially HLA-matched specimens reduces the likelihood of contamination. This strategy has proven useful in samples that had allele dropout or unanticipated homozygous typing. Repeat NGS library preparation consistently fixed the issues, indicating the problem is with library preparation rather than vendor related or sequencing.

4.2. Analytic challenges

Analytic challenges include determination of HLA alleles susceptible to allelic dropout (mostly HLA-DQB1), rare alleles, novel alleles, and homozygous samples. Homozygous specimens are especially difficult given the sensitivity of NGS to detect small amounts of DNA. An example of NGS sensitivity was a specimen typed as DRB3; however, based on DR-DQ association, DRB3 should be absent. Subsequent testing by sequence-specific oligonucleotide (SSO) probes confirmed the absence of DRB3. The contamination was traced back to the DNA extraction method and was likely from aerosolization of a neighboring sample. This DNA extraction method is no longer in use. Another specimen likely contaminated during library preparation by a neighboring sample, was also identified by DR-DQ association. These observations, while rare, identified weaknesses in technique or the assay that needed to be addressed to assure high-quality HLA typing results. The latter issue was addressed by implementing strict HLA-specific data quality criteria (Table 2) that encompassed depth of coverage (DOC), quality scores, and allele phasing in addition to our NGS sequencing criteria (Table 3). Depth of coverage had the greatest impact on HLA typing with as few as 67 reads able to provide accurate results [10].

Developing solutions to these challenges occurred through our validation by comparing the minimum DOC and other parameters necessary for accurate HLA typing. The details of how those criteria were developed will be published in *Journal of Molecular Diagnostics* summer 2016 [10].

Another challenge is staff training. Do not underestimate the amount of time needed to adequately train staff. Training was divided into two parts: technical assay and analysis. Each technologist had to have at least 2 successful NGS runs (without contamination) with >90% of the specimens they processed passing QC before they were considered technically proficient. For analysis, each technologist was given 20 blinded patient FASTQ files for analysis. Those 20 patients included several ways a specimen might fail and examples of allelic dropout. Three of the 20 patients were used for comparison purposes to determine competency.

4.3. Post-analytic challenges

A typical NGS run on the MiSeq platform generates about 18 gigabytes (GB) of total data. Most of the data is in the form of raw ∗.bcl files generated by the sequencer during each cycle and demultiplexed FASTQ files. A clinical laboratory needs to save the FASTQ files, InterOp folder, runinfo, run parameters, and patient analyzed data. These files account for approximately 7 GB of data when using a standard flow cell on the MiSeq platform and are required to be stored long-term. Each NGS platform will generate varying amount of data; however, the need for a long-term storage solution is consistent. To address data storage, laboratories should work with their information technology departments to obtain a secure, hospital-controlled storage (4 TB) for long-term storage that is backed up daily. The amount of storage required depends on the number of NGS runs expected and the length of time raw data are stored (minimum of 2 years, ASHI standard). An alternative to onsite storage is cloud-based storage of data, which has varying pricing options depending on vendor and amount of storage required. Unfortunately, many hospitals are avoiding cloud-based storage due to patient safety concerns.

Clinical application of NGS for HLA typing not only generates data storage challenges, but also turn-around time (TAT) challenges. We initially switched only HLA typing for hematopoietic stem cell transplant patient to NGS and kept sequence-specific oligonucleotide (SSO) for solid organ patient HLA typing. However, given our staffing (5 technologists) and workflow, within 6 months all patient HLA typing was performed using NGS. Only serologic

Table 1
QA measures to monitor NGS for HLA typing.

QA parameters to monitor[a]		
Pre-analytic	Analytic	Post-analytic
DNA quantity, amplification rate, library size	Allele dropouts, library quality, depth of coverage	NGS HLA typing failures TAT, repeating testing rate

[a] Assay dependent.

Table 2
UNC HLA laboratory HLA-specific data quality criteria.

UNC criteria for HLA typing	
% of reads >Q30	⩾80%
Phasing mismatches	0
Depth of coverage	⩾70
Core exon mismatches	0[a]
All other exon mismatches	0[a]
Intronic or UTR mismatches	<2[a]

UTR = untranslated region.
[a] Exception to the criteria are novel alleles.

Table 3
UNC HLA laboratory MiSeq run acceptance criteria.

UNC criteria for an acceptable MiSeq run			
Cluster density	% Cluster PF	% reads ⩾Q30	Error rate
639–1452	⩾75	>85	<1.54%

equivalents are reported for solid organ patients. We retain the high-resolution HLA typing information for use in virtual cross-matching and single antigen bead interpretation. Since implementing NGS our TAT was decreased by approximately 3 days compared to using Sanger sequencing [10].

4.3.1. Novel alleles

Since implementing NGS (July 2015), we have seen approximately 5 novel alleles per month. Most (35/50, 70%) of the novel alleles are single nucleotide variants (SNV) outside the antigen binding regions for HLA class I and II. The majority of the novel alleles are DPA1 (17/50, 34%) and C (10/50, 20%) alleles. Of note, 4 of the 10 novel C alleles are the same mutation found in 4 different patients. We have successfully named 4 new HLA alleles and are in the process of naming others, particularly the new HLA-C allele mentioned above [15]. The submission process to IMGT for naming is outlined on their website and requires additional experimentation to confirm the SNV. IMGT only requires sequence data from exons 2 and 3 for an HLA class I allele and exon 2 for an HLA class II allele. Importantly, when submitting alleles for naming, as much sequence data as possible should be submitted to prevent additional ambiguities from being generated. Identification of novel alleles is important because it may produce a null allele and impact HLA matching for hematopoietic stem cell transplantation or alter the ability to generate HLA antibodies. In our laboratory, all potential novel alleles where the SNV is within an exon are assessed for the production of a new null allele by analyzing the amino acid change. If the SNV occurs within an intron, the 5′ and 3′ sequences are analyzed for deviation from the conserved sequences necessary for splicing. Approximately 10% of pathogenic mutations are SNV that affect splice site [16,17]. Table 4 summarizes the reporting convention UNC has taken to report novel alleles.

5. Conclusion

NGS can reduce the ambiguity rate, cost, and TAT for HLA typing [6,7,9–11]. Validation of NGS for clinical HLA typing is challenging due to the numerous issues, including: sample types, complexity of the HLA genes, reliance on software for accurate HLA typing, and many more. Our policies to handle the aforementioned challenges were developed throughout the validation (9 months) with the exception of the novel allele policy. This review describes one approach to validating NGS for HLA typing, challenges faced throughout the validation and subsequent implementation, and ways to address those challenges. Like any new application of technology, the best policies and practices for NGS for HLA typing are evolving. Open discussion among regulatory agencies (ASHI, CAP, FDA) and clinical laboratories will facilitate standardization and implementation of newer technologies. HLA typing by NGS is allowing the histocompatibility and immunogenetics fields to ask new and important questions regarding HLA gene expression, regulation, and their impact on patient outcomes. These exciting areas will drive the future not only the HLA field but also improvements in patient HLA matching.

Table 4
UNC HLA laboratory policy for reporting novel alleles.

Amino acid change		Mutation location		
		Core exons	Non-core exons	Non-coding regions
Yes	No stop codon	Allele Group: XX	If G group is in IMGT, call G group. If not, Allele Group:XX	Does it alter conserved 5′ GT or 3′ CAG? No: two-field resolution. Yes: at Director's discretion
	Stop codon generated	Allele Group:XX (Novel null allele)		
No		Two-field resolution		

Allele group refers to the group of HLA alleles that typically corresponds to the serological antigen pertaining to a specific allotype. For example HLA-B*44:02:01 and HLA-B*44:03:01 both are within the HLA-B*44 allele group although they are encode different HLA-B proteins.

Acknowledgements

I would like to acknowledge Dr. John Schmitz for careful review and many discussions on this topic. I would like to thank Dr. Kristin Weimer for careful review of the article and the entire UNC HLA laboratory for their efforts to implement NGS within our laboratory.

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