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Point-counterpoint series: Confirmation of homozygous HLA alleles: Is it a necessity?

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ABSTRACT

We are pleased to introduce the new series in *Human Immunology* entitled "Point-Counterpoint." Within the field of HLA, there has been substantial change and evolution of technology that has greatly impacted the service HLA laboratories provide to providers and patients.

Recently, ASHI staff sent out a survey to all ASHI Laboratory Directors with the goal of finding areas within Serology and Molecular HLA testing where laboratory practices differed among histocompatibility laboratories. The data from the survey will be made available to the ASHI community at-large through ASHI Quarterly. Drs. Nicole Valenzuela and Eric Weimer have been using the data from this survey to determine relevant topics for this occasional Point-Counterpoint series.

In each issue, there will be two senior authors who are taking opposing views on a particular laboratory practice. The objective of the series is to curate expert viewpoints about what the literature currently supports in Immunogenetics and histocompatibility testing, and to identify key evidence that is lacking in controversial areas, to spur future research. Each expert contributor will present their logic and data behind their individual laboratory practice, with the intent of educating the greater ASHI community as a whole about variances in laboratory practices and why particular practices are performed.

The first series concerns the confirmation of homozygous HLA alleles. Subsequent articles will cover other key areas identified as variable in practice or controversial by the laboratory member survey. If there are particular areas of interest or practices the community feel should be debated, please feel free to propose such topics to Drs. Valenzuela or Weimer.

Point

In 2015 many HLA laboratories began evaluating the use of next-generation sequencing technology for HLA genotyping. To address the complex issues regarding validation, QA/QC, and implementation of NGS for HLA genotyping the American Society of Histocompatibility and Immunogenetics (ASHI) held an NGS Workshop in Dallas, TX. The main concern from the majority of the participants was allele dropout. Allele dropout is when a particular HLA locus is heterozygous for an allele but the technology/assay used identifies only a single allele, as if it were homozygous. HLA typing by NGS may be more prone to allele dropout compared to Sanger sequencing given the reliance on long-range PCR for HLA amplification (not all commercial assays) and the complexity of library preparation. The amplification step enables preferential amplification of one allele over another. Additionally, the amount of starting material necessary for amplification is lower with NGS compared to Sanger sequencing. However, those points are counteracted by the higher sensitivity of NGS compared to Sanger sequencing. Other lower resolution technologies, such as SSO (sequence specific oligonucleotide probe) and SSP (sequence specific primer), are less susceptible to allele dropout since they are typically designed to detect HLA antigen groups.

Now three years into the implementation of NGS for HLA genotyping, many laboratories are routinely processing samples through various commercial workflows with a high degree of success and significantly lower ambiguities and costs [1–7]. To first address the concern about allele dropout, an individual laboratory may consider how prevalent homozygosity is among their patient population. Examining the rate of homozygosity at University of

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Table 1
Number and Frequency of Homozygous HLA alleles.

	A	B	C	DRB1	DQB1
Total Homozygous	176	85	115	124	143
Overall Total Homozygous			643		
Frequency of Homozygosity	27.4%	15.6%	17.9%	19.3%	22.2%

North Carolina (UNC) since the implementation of NGS (2015) revealed the information below (Table 1).

At the University of North Carolina HLA laboratory, our initial NGS validation (compared to Sanger sequencing) using TruSight HLA v1.0 (2014–2015) demonstrated that allele dropout occurred in approximately 1% of samples (3/290 total samples). In 2016, UNC validated an updated version of the NGS assay and observed one sample with allele dropout (HLA-A) in 64 samples (1.6%). However, this locus was flagged by the analysis software for review and didn't provide a genotype unless the user made at least two edits. Many of these allele dropouts were in the initial implementation phase of NGS in the laboratory and due to our inexperience. To address the issue of allele dropout, which, based on our experience, is almost exclusively due to sample preparation errors and not lack of primer binding [4], some laboratories utilize dual analysis platforms. Use of multiple analysis software can aid in identification of incorrect HLA genotypes, as Gandhi et al. demonstrated use of multiple analysis software increased HLA genotype accuracy from 97.7 to 99.% [2]. As our experience with NGS has increased and the assay has been updated to address troublesome loci, DQB1 in particular, our rate of allele dropout has decreased. Examining the rate of allele dropout from April 2015 to October 2018, UNC has experienced 6 instances of allele dropout. The allele dropouts were identified by HLA associations, initial HLA genotyping, or related patient HLA genotypes. One was within HLA-B, two were in HLA-C, one in HLA-DRB1, and 2 in HLA-DQB1. These dropouts represented approximately 0.30% (vs 1% from validation) of all samples which have at least two samples received for HLA genotyping (initial and confirmatory). However, those numbers are artificially low since the majority of samples are heterozygous for a particular locus, as indicated by the data in Table 1. If you examine only samples which were homozygous for particular loci the frequency of allele dropout increases to 1.2%, 1.7%, 0.8%, and 1.4% of all homozygous genotypes within their respective loci. Thus, the true representation of overall allele dropout is 0.9% (6/643 homozygous samples). For comparison, Gandhi et al. found 2 out of 1891 homozygous samples had allele dropout using a different commercial NGS assay [8]. Additionally, Yin et al. found allele dropout occurred at 0.35% of *all* buccal swab samples [9]. The authors did not differentiate the number of allele dropouts from homozygous HLA genotyping. Since no allele dropouts were observed in blood samples, the authors concluded that primer-independent issues were likely contributing to buccal-derived sample allele dropout. Consistent with this hypothesis, repeating samples which had allele dropout (blood or buccal-based) corrected the allele dropout, suggesting the issue was in sample preparation or handling [4,10]. Currently, the laboratory is performing a direct 100 sample comparison between TruSight HLA v2, MiaFora, and AllType. From this evaluation, we can comment on the rate of allele dropout across several commercial NGS products for HLA genotyping. In this study, the number and frequency of samples in which allele dropout occurred from TruSight HLA v2, MiaFora, and Alltype, respectively, is shown in Table 2 (manuscript in preparation).

Table 2
Number and Frequency of allele dropout from commercial NGS HLA genotyping assays.

	TruSight	AllType	MiaFora
A*	0.0% (0/90)	0.0% (0/90)	1.1% (1/90)
B*	0.0% (0/95)	0.0% (0/95)	0.0% (0/95)
C*	0.0% (0/94)	0.0% (0/94)	0.0% (0/94)
DPA1*	0.0% (0/56)	0.0% (0/56)	0.0% (0/56)
DPB1*	0.0% (0/78)	0.0% (0/78)	0.0% (0/78)
DQA1*	0.0% (0/87)	0.0% (0/87)	4.6% (4/87)
DQB1*	0.0% (0/93)	0.0% (0/93)	0.0% (0/93)
DRB1*	0.0% (0/91)	1.1% (1/91)	1.1% (1/91)
DRB3*	0.0% (0/80)	0.0% (0/80)	0.0% (0/80)
DRB4*	0.0% (0/44)	0.0% (0/44)	4.5% (2/44)
DRB5*	0.0% (0/31)	0.0% (0/31)	0.0% (0/31)

Of the samples that had allele dropout, repeating the samples with PCR corrected the allele dropout. This suggested that the issue was in the NGS library preparation of the sample (potentially magnetic bead handling), not the assay. The observation of repeating samples to determine if allele dropout occurred has continued to part of our laboratory policy and has corrected any samples with suspected dropout. As others have published, specimen quantity and quality often have an impact on HLA genotyping results [9]. Consistent with this premise, Montgomery et al. demonstrated that DNA fragmentation significantly correlated with likelihood of HLA genotyping success by NGS [10]. Additionally, our laboratory uses all available information to assess the accuracy of the HLA genotype obtained. This includes using sample quantity/quality, HLA associations, initial HLA genotyping, or related patient HLA genotypes. Using these parameters has identified all instances of allele dropout. The accuracy of this approach was confirmed by CAP and ASHI proficiency testing (PT), where our laboratory has not missed a high-resolution PT sample typing since 2015 for all HLA loci (including DQA1 and DPA1). Since confirmatory HLA genotyping is performed prior to hematopoietic cell transplantation (HCT), there is an additional sample to confirm previous HLA genotyping results, often temporally close. Of the 287 confirmatory genotypings performed, only 4 required repeat testing prior to releasing HLA genotyping results. None of the repeats were due to suspected allele dropout on either sample (initial or confirmatory). The majority of the repeats were due to low specimen quantity or poor quality (mostly buccal swabs). The overall agreement between initial and confirmatory HLA genotypings without additional repeat or additional testing was 98.6% (283/287).

Application of the long-standing biologic principle of HLA linkage disequilibrium provides a key differentiator in identifying allele dropout. As evidence of how influential this relatively basic tool can be to identify allele dropout or other HLA genotyping issues, almost all commercial bioinformatics programs for HLA genotyping have incorporated HLA associations and haplotype analysis into their QA/QC parameters. However, HLA associations are primarily known for HLA-B/HLA-C and HLA-DRB1/DRB3/DRB4/DRB5/DQB1/DQA1, which doesn't provide additional QA/QC for the other HLA loci. For the other loci, utilization of public datasets has proven very useful. Our laboratory routinely uses allelefrequencies.net as a resource to identify observed class I/II haplotypes. Additional public resources are available from the 17th International Workshop (http://17ihiw.org/17th-ihiw-ngs-hla-data/) and published literature to enhance haplotype analysis. Haplotype analysis ensures that other HLA loci are part of the QA/QC process and it is our hope that commercial bioinformatics program will incorporate this type of QA/QC metric in the future.

Lastly, the accuracy of NGS for HLA genotyping was also demonstrated by the confirmation of rare HLA alleles. Of the 20 rare HLA alleles our assay has identified, all were proven accurate with additional testing. Our laboratory no longer performs this confirmation, but can send the DNA to another laboratory if necessary.

Our experience over more than 2 years has demonstrated that the risk for allele dropout is low (approximately 0.9%) but not zero. By routinely adhering to conservative QC parameters and applying known HLA haplotyping associations, our laboratory has been able to mitigate the risk of allele dropout. As with any clinical laboratory test, there is always some risk of error or mistyping. Allele dropout is no different. Confirmation of homozygous HLA alleles by alternative methods is excessive and unnecessary in the majority of cases. However, cases should be evaluated with all information available to assess quality of HLA genotyping results.

Eric T. Weimer

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Counterpoint

Rapid and continuous advancement in Next-Generation Sequencing (NGS) technologies has rendered a previously complex and expensive technique accessible to a wide range of research and clinical applications. NGS platforms have become ubiquitous in genomic laboratories and, with the adoption of NGS standards by the American Society for Histocompatibility and Immunogenetics (ASHI) in 2016, this technology is now increasingly employed by clinical HLA laboratories [1,2]. Clinical diagnostic laboratories implement rigorous quality control procedures for NGS typing but, as with all tests, artifacts occur. The most commonly reported are due to sample contamination, sequencing errors, read misalignments, or polymerase chain reaction (PCR) amplification biases, and the most concerning are allelic dropouts (ADO) [3]. For the purpose of clarity, this editorial will focus on technical concerns of ADO, specifically 'silent' ones that are technically valid but erroneous.

Although PCR amplification is considered extremely robust and is a mainstay technique in any molecular laboratory, ADO where one or both allelic copies at a locus fails to amplify during PCR, has long been recognized as an important problem [4,5]. In fact, the 2012 Clinical Laboratory Standard Institute guidelines state that assay development and quality control should include measures aimed at both detecting ADO and minimizing its occurrence [6]. In most standard genotyping scenarios, ADO translates straightforwardly to erroneous homozygotes. With HLA, the situation becomes much more complicated [7].

Allelic dropout occurs either due to sequence independent factors or allele-specific sequence variations. Poor sample quality [8,9], low DNA input concentration [10], PCR conditions [3], DNA polymerase fidelity [11–13], polymerase-blocking secondary structures [14] or the presence of inhibitors [15] may cause an allele to be incompletely amplified. Alleles with low expression levels may also present a potential problem [16], and the seldom-quantified "human error" contributes hugely to genotyping errors [17]. These situations usually affect either of the two alleles with equal probability, are random and usually not reproducible. Technical or biological replicates usually resolve the artifact.

Non-random or allele-specific dropout is usually sequence dependent. If a variant is located in a primer binding site, primer hybridization efficiency is affected and can lead to failed amplification and allele bias [18,19]. Common variants include the presence of one or more SNPs, especially towards the 3' end of the primer [18], transitional mutations in the primer region [20] or indels [3,15]. The problem is further exacerbated when mismatched primers systematically penalise specific alleles (15). Stochastic fluctuation (sampling error) due to an initial low sampling of a heterozygous sample can also result in what appears to be preferential amplification [3]. In such cases, running technical duplicates will not change the conditions of the initial PCR bias, confirming what appears to be valid homozygous results.

Molecular HLA typing techniques have replaced serological methods in most histocompatibility laboratories and provide more precise clinical results. Allele dropout is not limited to NGS technology; any amplicon-based application for targeted re-sequencing is vulnerable to this problem. Sequence-specific oligonucleotide probes (SSOP), sequence-specific primers (SSP) and sequencing-based typing (SBT) are all dependent on PCR and assume that biallelic amplification has occurred. Since many clinical molecular diagnostic laboratories do not systematically test each locus and allele by multiple assays, data as to the incidence of allele dropout rates using SSOP/SSP is limited. Dropouts have been reported in both class I and II loci in SSOP [21]. Specific allelic issues have been identified and published [22–24] but these were caused by specific primer mismatch for a particular allele combination, as opposed to a systematic problem with biased amplification. Sanger sequencing [24–26] has been reported to be the most sensitive to ADO artifact, probably because of its specific sequencing primers in contrast to the more generic primers used in SSP and SSOP. SSP is least likely to be affected by this phenomenon due to multiple primers targeting different binding sites in each well

[27]. It would appear that no (current) technology is immune to the probability of ADO. Perhaps surprisingly, testing across multiple assays does not always reveal a dropout [28,29]. In both of these reported cases, the silent dropped allele was not noticed until verification or sibling data was available. Such insidious dropped alleles are extremely difficult to detect and are a significant and real concern. In cases where sibling/parental typings are unavailable, or for solid organ recipients (or donors) where confirmatory typings are not required, such silent dropped alleles may be missed completely.

Although the complex pattern of patchwork polymorphisms in HLA class I and class II genes has made, and continues to make, high-resolution HLA typing challenging, the technology has been shown to be robust and capable of providing single-pass allele level typing with minimal ambiguity [30–34]. There are several commercially available amplification-based HLA typing kits such as the AllType from Thermo Fisher/Life Technologies (Carlsbad, CA, USA), HLA Holotype from Omixon (Boston, MA, USA) NGSgo from GenDx (Utrecht, Netherlands), MiaFora from Immucor (Norcross, GA, USA) and TruSight HLA from Illumina (San Diego, CA, USA). These platforms all rely on a preliminary amplification step of genomic DNA based on the PCR, to both target and enrich for the HLA gene cluster. For amplification to be both successful and efficient, the hybridization reaction typically requires nearly perfect target/primer sequence complementarity. In non–HLA situations, such as in cancer diagnostics, primer design can be guided by a reference sequence, so known single nucleotide polymorphisms (SNPs) can be avoided. However, this cannot be achieved when designing primers for the highly polymorphic HLA regions. The extreme diversity of the HLA genes, compounded by the need to use long-range PCR for longer amplicons for NGS library preparation, increases the likelihood of ADO artifact formation. The ADO rate in NGS technologies has been assessed more thoroughly than other molecular HLA typing technology, perhaps due to the ease with which multiple samples can be typed in a single run. The largest evaluation of ADO rates in NGS was carried out by Yin, et al., who performed parallel SSO typing of all homozygous alleles assigned by NGS from over 10,000 buccal samples [21]. Overall, an allele dropout rate of 0.35% was observed based on confirmatory typing of 6,790 homozygous alleles. Interestingly, no allele dropouts were detected in the 122 blood-based specimens, and the authors concluded that DNA quality/quantity may have influenced the ADO rate in buccal samples. In contrast, Ehrenber et al. did not observe any allele dropouts associated with allelic imbalance [35]. Although most cases of allele dropouts are likely PCR-related and generally can be considered extreme cases of allele imbalance, our laboratory as well as others have observed false homozygous HLA typings due to chromosome 6 aberrations in particular blood cancers (e.g., acute lymphocytic leukemia) [36–38]. A true loss of homozygosity (LOH) is distinct from assay-related ADO, although both result in an erroneous homozygous typing. In bone marrow patients, this may be circumvented by using buccal swabs to confirm typing, although DNA degradation of buccal swabs tends to be greater than from blood samples and would, therefore, decrease the PCR efficiency.

Data collected from our laboratory from the first six months of 2018 shows that 1119 patients were HLA typed using the Omixon Holotype V2 kit. Of these, 329 patients required at least one other test in order to obtain full 11-loci typing (SSO or SSP) and 790patients were fully typed at all 11-loci by NGS and required no further testing. From our validation of the Holotype kit, we were aware of the propensity of poor amplification of the DQB1*03 alleles. Our approach has therefore been to automatically reflex test all DQB1 homozygous typings for confirmation. Of the 1119 patients typed, 128 appeared homozygous at DQB1, of which 121 were reflex tested for DQB1. A total of 13 samples were confirmed as DQB1 drop-outs, all of which were the 03 allele group (10.2%). There did not seem to be a correlation with the second allele, nor with any sample characteristics such as input DNA concentration. All 13 samples had a median concentration of 27.98 ng/ul (range 16.1–56), which is well within the acceptable range for our protocol (15–35 ng/ul). Of the 13 drop-out cases, 3 were bone-marrow donors, 3 were bone-marrow patients, 6 were kidney recipient and 1 was a deceased donor (7 Male:4 Female:2 unknown). In all 13 cases, quality control metrics were all within range during library preparation, however the second allele had in fact failed to amplify. Amplicon quantification was not diagnostic in identifying an amplification issue, since the preferred allele was amplified sufficiently as to mask the 'loss' of the second allele. Repeated NGS did not resolve the second allele. Using association information, 9 of the associations were very common, 3 were common and 1 was rare, suggesting that 9 of the erroneous homozygous typings could have been identified and corrected but 4 cases would still have potentially been incorrect. From all 128 homozygous DQB1 typings, 3 were rare and 5 had unknown associations, which suggests that even using known association information to identify erroneous homozygous typings, we could still have mistyped 8 cases, a 6.25% miss rate. Our ADO rate of 10.2% is significantly higher that has been observed in other labs (i.e. Yin et al. [21]). One possible explanation for this discrepancy could be due to manual vs automated library preparation. Liquid handlers programmed for NGS library preparation protocols have a coefficient of variance (CV) on pipetted volumes as low as 0.3% [39], while human users can have CV's of up to 15% (correspondence data).

We postulated that there must be at least 1 SNP, if not more, on the DQB1*03 allele which directly influences the annealing efficiency when in the presence of another allele. Software detection limits of allele imbalance may impact whether or not the minor allele was detected at all, as less than 1.5%–10% of the background sequence can be assigned to another allele by these new sequencing techniques [40–42]. However, our experience for all cases of confirmed dropout was that the second allele was not present in the data, even when we manually visualized in the alignment browser, suggesting these were true assay-dependent dropouts, likely at the amplification stage. This was also supported by the fact that amplicon quantification did not show lower level of amplification, as would be expected if there was poor amplification. Rather, a bias against the DQB1*03 allele, which would still allow the major allele to amplify normally, is the most likely cause. The choice of DNA polymerase may also impact the efficiency of amplification of both DQB alleles [43,44]. To this end, Omixon V3 kits have improved both the DQB1 primers and optimized the polymerase used in the Holotype amplification, as well as updating the HLA-Twin HLA-typing software. Assay-related issues have also been published by other laboratories [45] including DQB1 [46], although in this case it was suboptimal detection of the DQB1*02 allele using laboratory developed primer sets.

The impact of allele dropout in molecular diagnostics cannot be underestimated, as it has been previously shown to affect genotyping results of several diagnostic assays including cystic fibrosis [47], tyrosinemia type 1 [14], muscular dystrophy type 1 [48], methylation [49] and Wilson disease [50]. However, in the case of histocompatibility diagnostics, a genotyping error such as this may have serious implications for both bone marrow transplants (increased likelihood of mismatching and GvHD) and solid organ transplants (potentially missed antibody specificity).

Bone marrow transplantation

Loss of heterozygosity (LOH) is a true 'dropout' phenomenon, unrelated to assay artifact and is more common than previously

expected. HLA aberrations in tumors (LOH in chromosomes 6) have been predicted to be present in 30–40% of all human cancers [51,52] and LOH is likely to be underreported in hematological malignancies [27,53]. Since acute leukemias and myelodysplastic syndromes (MDS) are the most common indications for allogeneic stem cell transplantation (HSCT), cases sent for testing may have an elevated risk of biological LOH.

In a biological sample which contains a high percentage of abnormal clones, it is conceivable that the DNA used for HLA genotyping is extracted from predominantly affected cells, leading to false homozygosity (or for that matter, erroneous HLA typing). If unnoticed, the patient may become an HLA-mismatched HSCT, which is known to carry a significantly increased risk of severe graft-vs-host disease and mortality [54–56]. In addition, if the LOH is not noticed immediately, the delay caused by reaching and typing new donors with the right HLA type may be detrimental to the patient, who may undergo additional rounds of treatment in the interim.

In fact, our laboratory has observed 2 confirmed true LOH cases (unpublished data) and our experience led us to modify our practices. Biological samples with large buffy coats are more likely to have abnormal clones and are flagged at the extraction stage. Increased communication and collaboration with the treating clinicians have been helpful, as knowledge of the diagnosis alerts us to patients at risk since particular hematological malignancies are more prone to LOH. Blood samples for the original and confirmatory HLA genotyping are frequently taken within a few days of each other and not uncommonly at the time of diagnosis when the proportion of malignant cells in blood is usually at its peak. Therefore, we also request a third confirming sample from the patient either after initial treatment (when the proportion of the leukemic cells has diminished) or when the disease is in remission. If possible, we request familial samples to determine inherited haplotypes to exclude the possibility of LOH. In addition, samples other than blood, i.e. buccal swab [38] or saliva have been used to minimize the risk of undetected LOH, although it should be noted that LOH may also appear in saliva samples [27].

Solid organ transplantation

Whilst high-level genetic compatibility is not the principal paradigm currently employed for matching of donors and recipients in Canada, accurate high-resolution typing of donors is essential to avoid the selection of recipients with pre-formed donor-specific antibodies (DSA). Missing a heterozygous allele could result in a recipient receiving an organ to which they have pre-formed DSA, a major cause of allograft loss [57–59].

Conclusion/closing argument

Allelic dropout (usually assay-related) and loss of heterozygosity (usually of biological origin) can complicate any current HLA typing technology, although SBT and NGS appear most susceptible to these artifacts which result in erroneous homozygous typings. With the possibility of a 0.4% biological LOH [27] and assay dropout rate of 10.2% on NGS technologies (our data), the risk of erroneous homozygous typings would justify developing a low-threshold protocol for retyping using a different method. Even when validating and controlling for DNA quality and quantity metrics, using association databases and being aware of the limitations of particular primer sets (commercial or otherwise), it is still possible that ADOs could occur. DNA quality and quantity can be variable and unavoidable. Designing SNP-free primers is complicated, fraught with technical intricacies and almost impossible. With the publication of association data for all HLA loci by the 17th International HLA and Immunogenetics Workshop, it is our recommendation to check any typings with non-typical associations [60]. One should be particularly careful in the presence of partial HLA homozygosity, even at the level of a single locus, especially for HSCT patients. Haplotypes which are formed by rare alleles or are atypical in the population should be confirmed, however in populations with extensive ethnic subgroups, this method of risk profiling may not be possible.

This review hopes to draw attention to one of the most underestimated limitations of genetic testing in general, for which medical and laboratory communities need to be aware. We highlight the risk of allele dropout artifact (and biological LOH) on diagnostic results and the importance to detect and circumvent it in clinical molecular diagnostics. It is therefore highly recommended that all HLA laboratories remain alert to ADO artifact, and form their own guidelines for appropriate screening.

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