VALIDATION OF HLA TYPING BY NGS

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CONFlict of Interest

I have financial relationship(s) with:
Advisory Board, Illumina

My presentation does include discussion of off-label use. Commercial reagents for HLA typing by NGS are labeled for research use only, however I will be discussing the clinical application of these reagents.

Overview

- Our validation approach
- Validation summary
  - QC
  - Analysis
- Things to consider
- Cost analysis
- Preparation of ASHI submission packet
Sanger → Next-generation sequencing (NGS)

**Sanger Sequencing**
- 2 chromosomes sequenced concurrently
- Highly polymorphic HLA region leads to ambiguities in allele determination
  - Leads to 53% repeating testing to resolve ambiguities

**NGS**
- Massive parallel sequencing
- Depth of coverage allows for accurate phasing of HLA alleles
- Reduced ambiguities (~93.5%)
- Mostly full gene sequencing

ASHI NGS Accreditation Process

- NGS is a new test SYSTEM
- 50 unique samples + 20 blinded samples
  - Minimum of 80% concordance (1st and 2nd field)
- Where are you going to get blinded samples?
- Sample types you routinely receive
- Sample NGS runs should closely mimic your expect clinical runs
- Precision studies
  - Inter- and intra-assay
- Follow the document available from ASHI for validation of new test system
- Collect all available information
  - Lot numbers & expiration dates of reagents
  - Generate worksheets that will be used for clinical testing
- Submit NGS validation packet to ASHI Commissioner

ASHI NGS Standards

- ALL other relevant standards apply
  - Competence
  - QC review and monitoring
- D.5.2.11 contains all NGS-specific Standards
  - D.5.2.11.1 Sufficient representation of all pertinent allelic specificities of the locus tested in order to evaluate possible allele dropouts
  - D.5.2.11.2 When barcodes are incorporated after target enrichment, fidelity of the barcoding method to identify a particular sample needs to be monitored (e.g., by rotating control samples with different barcode sequences)
  - D.5.2.11.4 Instrument performance measures must include data from internal control samples and/or vendor supplied quality control material
Validation Approach

- Utilized previously high-resolution typed samples for HLA-
  - A
  - B
  - C
  - DRB1/3/4/5
  - DQB1
  - DQA1
  - DPB1
  - DPA1
- Validation of all HLA loci
- Use of NGS for solid organ HLA typing
- Run size should mimic your expect volumes
- Utilize previous typed sample on every run
  - QC of indices (ASHI Standard)
  - QC of MiSeq cartridge and flow cells (one-off reagents)
- Controls
  - PhiX (Illumina sequencers)
  - Sequencing control
  - National Institute of Standards & Technology (NIST)
  - Reference material 8398
  - CDC Reference materials

NGS Validation Timeline

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>11/2014</td>
<td>Initial run with mix of commercial NGS reagents</td>
</tr>
<tr>
<td>12/2015</td>
<td>More commercial kit</td>
</tr>
<tr>
<td>4/2015</td>
<td>Submission of packet to ASHI Commissioner for interim blinded samples</td>
</tr>
<tr>
<td>6/2015</td>
<td>Completion of blinded samples</td>
</tr>
<tr>
<td>7/2015</td>
<td>Updated packet with more blinded samples</td>
</tr>
<tr>
<td>8/2015</td>
<td>Updated packet with more blinded samples</td>
</tr>
</tbody>
</table>

Impact of Sample Type and Concentration on HLA Typing
**Validation Numbers**

<table>
<thead>
<tr>
<th>Sample</th>
<th>HLA Class</th>
<th>Gene</th>
<th>N</th>
<th>Allele Dropout %</th>
<th>Success %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>HLA-A</td>
<td>A1</td>
<td>10</td>
<td>50</td>
<td>99</td>
</tr>
<tr>
<td>Sample 2</td>
<td>HLA-B</td>
<td>B1</td>
<td>15</td>
<td>60</td>
<td>99</td>
</tr>
<tr>
<td>Sample 3</td>
<td>HLA-C</td>
<td>C1</td>
<td>20</td>
<td>50</td>
<td>99</td>
</tr>
</tbody>
</table>

**Validation Summary**

<table>
<thead>
<tr>
<th>Overall Numbers</th>
<th>Blinded Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coding</td>
<td>No Coding</td>
</tr>
<tr>
<td>HLA-A</td>
<td>67</td>
</tr>
<tr>
<td>HLA-B</td>
<td>60</td>
</tr>
<tr>
<td>HLA-C</td>
<td>55</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>53</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>80</td>
</tr>
<tr>
<td>HLA-DP</td>
<td>80</td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>80</td>
</tr>
<tr>
<td>HLA-DRB3</td>
<td>80</td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>80</td>
</tr>
</tbody>
</table>

**Impact of Sample Quality on HLA Typing**

- **Agilent TapeStation 4200**
- **Genomic DNA assay**
- **DNA Integrity Number (DIN)**
- **Agilent TapeStation 4200**
- **Fragmentation measurement of BS key predictor to allele dropout**
- **DNA concentration independent**
Validation Summary

**Overall Numbers**

<table>
<thead>
<tr>
<th>HLA Typing QC</th>
<th>DQA1</th>
<th>DQB1</th>
<th>DRB1</th>
<th>DRB5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test allele</td>
<td>XLR</td>
<td>XLR</td>
<td>XLR</td>
<td>XLR</td>
</tr>
<tr>
<td>Leaky allele</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Mostly due to contamination
Some allele dropout

Depth of Coverage and Quality Scores

- **HLA typing QC**
  - Known HLA typed sample
  - QC of sequencing reagents
  - Quality metrics of individual HLA loci
    - **Coverage**
    - Q30 scores

- QC Reins Supreme
  - Two tiered QC scheme
  - Sequencing QC
    - Cluster Density
    - Clusters passing filter (PF)
    - Format of reads ≥Q30
    - Read error rate
  - Rotation of the known HLA typed sample with barcodes
    - Ensures fidelity of index primers
### HLA Specific Quality Metrics

<table>
<thead>
<tr>
<th>Criteria for HLA typing call</th>
<th>% of reads &gt;=Q30</th>
<th>&gt; 80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phasing Mismatches</td>
<td></td>
<td>&lt; 0</td>
</tr>
<tr>
<td>Depth of coverage</td>
<td></td>
<td>≥ 70</td>
</tr>
<tr>
<td>Core Exon Mismatches</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>All other exon mismatches</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Accurately called HLA typing results: 50 depth of coverage, 74% of reads ≥Q30

### NGS Analysis

- Independent validation of software
  - Includes MiSeq Controller and Reporter software
  - Software updates

- If any question regarding HLA type, repeat using Sanger sequencing or lower-resolution technology (depends on issue)
  - Rare allele confirmation
  - Mostly used to help staff increase comfort level

### NGS Lessons Learned

- How are you going to store the data?
  - Each run generates ~8 GB of total data

- Know your assay!
  - Homozygous samples can be challenging
  - Limit of detection (allele imbalance)

- Check DR-DQ and B-C associations
  - Improves confidence in NGS typing results
  - Aids in determination of allele drop-outs or potential contamination

- Understand novel alleles
  - How will you report them?
Things to Consider

- Are you going to maximize cost effectiveness or turn-around time?
- Automation? Which steps?
  - Requires another validation
  - ASHI standards regarding pre- and post-amplification areas
- Will you change how you bill patients?
  - CPT codes are for high- or low-resolution HLA typing
  - NOT technology based

NGS Cost Analysis

- Sanger
  - 53% of samples require additional testing
  - $80/loci (full HLA typing)
    - Includes repeat testing and supplies
- NGS
  - Estimated 3% ambiguity rate
  - $50/loci (full HLA typing)
    - Includes estimated repeat testing and supplies

ASHI NGS Accreditation Process

- Staff training
  - How will you ensure staff is adequately trained to perform the assay?
  - Who will analyze the results?
  - How will you train and perform competency assessment?
- Analysis
  - What are the criteria for a “successful” NGS run?
  - What are the metrics that will be used to make HLA typings calls?
ASHI NGS Accreditation Process

- Compile all worksheets
  - Lot number and expiration dates
- Compile all new procedures and policies
  - Have to be signed off by the Lab Director
- Provide rational for your policies
  - How will you prevent false negative (or false positive) HLA typing results?

ASHI NGS Validation Summary

- Follow instructions on for new TEST SYSTEM
- Think about your processes
  - Where does NGS fit in?
- Plan your overall strategy
  - New procedures and/or policies
  - IT infrastructure
  - Develop and collect worksheets
- Keep log of samples that have issues