Second Webinar Session

A second session of this webinar will be hosted

**Wednesday, June 28**

2:00 PM EST  
(1800 GMT)

Register at the link below:

https://attendee.gotowebinar.com/rt/8827305241673767681

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Upcoming Webinar

Join Us for a Webinar

Association of Hemoglobin A Clearance and RBC Antibodies in Chronically Transfused Children with Sickle Cell Anemia

Registering:
Marlane E. Yen, MD, MSc
Assistant Professor of Medicine (Hematology/Oncology)
Children’s Healthcare of Atlanta

Moderator:
Dr. Kassadie Jackson, MD - Blood Transfusion Services

July 12, 2017: 8:00 am and 2:00 pm EDT  
(12:00 and 18:00 GMT)

To RSVP for the webinar, call 844-700-0020 or email us at Marketing@Immucor.com

https://attendee.gotowebinar.com/rt/8827305241673767681
Handouts


Continuing Education

• PACE, Florida and California DHS
• 1.0 Contact Hours
• Each attendee must register to receive CE at: https://www.surveymonkey.com/r/HLA_NGS_June2017
• Registration deadline is July 14, 2017
• Certificates will be sent via email only to those who have registered by July 28, 2017

Presentation Recording

• Session will be recorded and posted.
  – Access information will be sent to each registrant when the recording becomes available
• No CE issued for participating in recording
Questions?

- You are all muted
- Q&A following session
  - Type in questions

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The opinions contained in this presentation are those of the presenter and do not necessarily reflect those of Immucor.
### Comparison of Current HLA Typing Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Coverage*</th>
<th>Allele Ambiguity**</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSO</td>
<td>Exon</td>
<td>Yes</td>
<td>Medium</td>
</tr>
<tr>
<td>SSP</td>
<td>Exon</td>
<td>Yes</td>
<td>Low to Medium</td>
</tr>
<tr>
<td>SBT (Sanger)</td>
<td>Exon</td>
<td>Yes</td>
<td>High</td>
</tr>
<tr>
<td><strong>Whole Gene NGS</strong></td>
<td><strong>Whole Gene</strong></td>
<td><strong>Few to None</strong></td>
<td><strong>Highest</strong></td>
</tr>
</tbody>
</table>

Coverage: The region of gene to be interrogated
Allele Ambiguity: Unresolved typing due to the lack of information from un-sequenced region

### Problems with Current HLA Typing Methods

Problem with Current HLA Typing Methods
- Partial Coverage of HLA gene
  - Makes assumptions in un-sequenced regions
  - Creates Typing Ambiguities
  - Wrong assumptions lead to undetected functional mismatch
  - Need confirmatory testing to resolve ambiguities
  - Each mismatch reduces transplant success by 4-6%
  - Long donor search time
  - Deterioration of Patient Status

### Solution to Current Problems with HLA Typing

- **How to solve current problems with HLA Typing?**
  - Sequence entire genomic DNA
  - Provide complete information of all relevant HLA genes
  - HLA typing with no ambiguities
    - Ability to identify new alleles
    - Ability to identify new null alleles
  - Shorter TAT and reduced cost by getting results in a single pass
  - Only whole gene NGS approach can provide the solution
Benefit of High Resolution Typing for Solid Organ Transplant

Primary cause of transplant rejection

Presence of DSA in patient's sera

Identification of permissive mismatch

HLA antibodies are specific for epitope

The same antigen identified based on low resolution typing can have different epitope repertoires; thus, different reactivity with DSA

Solution?

High-Resolution Typing

Identify specific epitope to find permissive mismatch for sensitized patients

Develop permissive mismatch strategy to prevent future sensitization for non-sensitized patients

Increase transplant opportunities

Improve long term graft survival

MIA FORA NGS FLEX HLA Typing Kit

MIA FORA NGS FLEX Kits

Fit Your Workflow

<table>
<thead>
<tr>
<th>KITS</th>
<th>CLASS I</th>
<th>CLASS II</th>
<th>KIT SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLEX 5</td>
<td>A,B,C</td>
<td>DQB1, DRB1</td>
<td>24 &amp; 96</td>
</tr>
<tr>
<td>FLEX 6</td>
<td>A,B,C</td>
<td>DQB1, DPB1, DRB1</td>
<td>24 &amp; 96</td>
</tr>
<tr>
<td>FLEX 9</td>
<td>A,B,C</td>
<td>DQB1, DRB1, DRB1/4/5</td>
<td>24 &amp; 96</td>
</tr>
<tr>
<td>FLEX 11</td>
<td>A,B,C</td>
<td>DQA1, DQB1, DRB1, DRB1, DRB1/4/5</td>
<td>24 &amp; 96</td>
</tr>
</tbody>
</table>

- Four different configurations
- 24 and 96 sample kits
- 8-96 samples/run
- Compatible with Illumina Miniseq, MiSeq and NextSeq
- Software version 3.0 including Windows and Linux Workstations

Confidential

MIA FORA

NGS

FLEX

• Four different configurations
• 24 and 96 sample kits
• 8-96 samples/run
• Compatible with Illumina Miniseq, MiSeq and NextSeq
• Software version 3.0 including Windows and Linux Workstations

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MIA FORA has The Highest Overall Coverage

Whole Gene Coverage

Exon 1 to Exon 6

MIA FORA Coverage

Coverage Comparisons*

<table>
<thead>
<tr>
<th>Core Reagents included in the kit</th>
<th>MIA FORA</th>
<th>Vendor 1</th>
<th>Vendor 2</th>
<th>Vendor 3</th>
<th>Vendor 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Range PCR primers</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Long Range PCR reagent</td>
<td>√</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Library Construction kit</td>
<td>√</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adaptor Index</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnetic Beads</td>
<td>√</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

MIA FORA NGS FLEX Kit
All Reagents Included in Kit

- Kit includes everything you need to construct sequencing library
- No need to purchase any core components separately

* As of June 2017
**MIA FORA FLEX Kit Components**

- **PCR Reagents**: 96-to-One™ pre-mixed master mix to cover appropriate genes.
- **Library Prep Reagents**: Reagents for fragmentation, End Repair, A Tailing, Adaptor Ligation, and library amplification.
- **Adaptor Plate**: Pre-aliquot Adaptor Index plate.
- **Magnetic Bead Kit**: Filled Vial of Agencourt AMPure XP bead.

**MIA FORA NGS FLEX Workflow**

- Long range PCR to library loading to the sequencer in less than 24 hours
- Sample to results in 48 hours

**Automation Options**

- Biomek and Hamilton Liquid Handler
  - MIA FORA offers optimized and validated automation protocols for both Pre- and Post-PCR steps.
MIA FORA Genotyping Strategies
Three Algorithms

MIA FORA
Two complementary Bioinformatics

Phasing
Mapping

Dynamic Phasing Analysis Algorithm*
Consensus Algorithms
Competitive Alignment Algorithm*

*Exclusive to MIA FORA NGS, Patent-pending

MIA FORA NGS software is the only software that utilizes three algorithms to determine HLA genotypes

High Accuracy of Automatic Assignment

Automatic Assignment Concordance (n=48)

Technical Support

Over 15 years of HLA experience in both serology assay and molecular assay support

Implementation Support
- Facilitate communication with vendors
- Confirmation of proper installation of all instrument
- Provide comprehensive implementation Guide
- Provide on-site training

Validation Support
- Provide comprehensive Validation Guide
- Provide checklist for ASHI/CAP/IFI accreditation
- Provide on-site consultation for validation

Continuous Technical Support
- On site and remote assay support and troubleshooting
- On site and remote Software and IT support and troubleshooting

Over 15 years of HLA experience in both serology assay and molecular assay support
### Future Direction of HLA Typing

### MIA FORA NGS FLEX KIT

**Summary**

1. **Short turnaround time**
   - Long range PCR to sequencing in less than 24 hours
   - Sample to result in 48 hours

2. **Flexible kit configurations**
   - Available in 5, 6, 9 and 11 gene sets; 24 and 96 test kits
   - Works on a variety of Illumina platforms and flow cells

3. **All reagents included in the kit**
   - No need to purchase long range PCR reagent, Adapter index plate, and/or Magnetic bead separately

4. **Streamlined, user-friendly process workflow**
   - Beckman Liquid Handler automation options available

5. **Software utilizes three algorithms**
   - Generate accurate typing results on the first pass

6. **Excellent Technical Support**
   - Provide industry-leading, best-in-class technical support

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### Future direction of HLA typing

- Current HLA typing methods (SSO, SSP, Sanger) will be replaced with NGS due to:
  - Lower cost
  - No ambiguity
  - Higher resolution
  - Higher accuracy

- Due to the lower cost of NGS compared to SSO and Sanger, both Bone Marrow Transplant and Solid Organ Transplant labs can benefit from implementing NGS

- SSO and SSP will be used for deceased donor typing
Other NGS Applications in HLA

- Identification of matching donor and recipient
- Monitoring chimerisms after bone marrow transplant
- Study genetic predisposition to auto-immune and inflammatory diseases
- Risk assessment of adverse drug reactions
- Population genetics studies
- Immunotherapy

Thank You!

Questions?

- You are all muted
- Q&A following session
  - Type in questions
Overview

- The lab has tried six different HLA NGS manufacturers
- All workflow estimates based on single 8 hour shifts
- Two manufacturers were not used beyond initial training runs
  - One excluded due to very cumbersome set up if doing more than 12 samples, required two techs for most of the protocol
  - Other excluded due to lack of customer service and flexibility in pricing
- The lab has done multiple clinical runs using kits from the four remaining manufacturers
  - Clinical runs consist of 30-48 samples

Work Flow of Manufacturer A

- Set up PCR by 10am (1 hour set up for up to 48 samples, under 2 hours cycling)
- Bench clean and quantify amplicons
- Library construction and bead clean
- Secondary amplification and bead clean
- Library quantification and pooling
- Isothermal amplification and enrichment
- Put on sequencer
- After 12 hours of sequencing time, start data processing
  - By mid-morning, techs analyze
  - Report 30+ samples by EOB

Option to do an extended 10.5 hour Day 2 with automated overnight replacement of isothermal amplification. Day 3 flow begins with putting the library on the sequencer.
### Advantages of Manufacturer A

- Initial amplification is simple and uses a minimal amount of DNA
- Poor samples (concentration/quality) still yield usable results
- Software is visually appealing and simple to use
- Can run between 8-96 samples
- Barcode flexibility allows for multiple runs of up to 96 samples on each run, barcode selection and manipulation is simple
- Only need two cyclers for initial PCR
- Few ambiguities
- Few allele dropouts

### Disadvantages of Manufacturer A

- Not a well defined kit – requires combining multiple kits for one run
  - Tracking lots and amounts left can be difficult (kits are designed for various sample numbers per run)
- Lots of hands on time, short incubations and multiple bead cleans
- Complex protocol requires some finesse
- Multiple sample and plate transfers can lead to error
- No excess product beyond pooling for protocol mishaps

### Work Flow of Manufacturer B

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>11 hours</td>
<td>40 hours</td>
</tr>
</tbody>
</table>

- Set up PCR (2.5 hour setup, 3 hour cycling)
- Selectively check amplicons on gel
- Pooling, cleanup, barcode, check on gel
- Secondary pooling, cleanup, purification
- Gel check, quantification, combine into 1 tube
- Load on sequencer
- Max 40 hours of sequencing time, start data processing
- Tech analyze
- Report 30+ samples by EOB
Advantages of Manufacturer B

- Uses very little DNA and works very well with low concentration samples
- Initial PCR is fast enough to allow for different day 1 configuration to compensate for longer sequencing time
- Targeted exon – gets exons other manufactures can’t (DPB1 exon 1)
- Data processing is very fast
- Reliable results

Disadvantages of Manufacturer B

- Complicated PCR set up (12 wells per sample)
- No correspondence between individual master mixes and loci (6 mm for each class not pertinent to specific loci)
  - If a sample fails for a particular amplification, can’t do selective Sanger
- Pooling of samples not as straight forward as other manufacturers
  - Different pools have different volumes / different amp
- Multiple gels to check amplifications – time consuming
- More ancillary reagents than MIA FORA and manufacturer C for library construction

Work Flow of Manufacturer C

- **Day 1**
  - Set up long range PCR to run overnight (2 hour set up time up to 48 samples with 6 hour cycle time)
- **Day 2**
  - Quantify amplicons
  - Library construction, quantification
  - Put on sequencer
- **Day 3**
  - After +/- 24 hour sequencing time, start data processing (can’t be started remotely, 10 minutes per sample)
- **Day 4**
  - Data available first thing in the morning
  - Techs analyze
  - Report 30+ samples by EOB

Extended shift of ~11 hours
Advantages of Manufacturer C

- Very easy library prep (many steps are mixing a buffer and an enzyme and adding to samples)
- Several long incubations allow techs to do other things
- All steps produce enough product to set up again in case of mishaps
- Run between 8 and 72 samples (given the limitation of our sequencer – with another model could run 96)
- Have not seen any allele dropouts that were not flagged by software
- Least expensive offering of their primer compared to others

Disadvantages of Manufacturer C

- Software is not intuitive, annoying to navigate, and not visually appealing
- DRB1 and DPB1 results can be inconsistent (messy DRB1, DPB1 is not a robust amplification)
- Has more ambiguities than some other manufacturers
- Long range PCR set up is more complex than manufacturer A or MIA FOR A
- Requires the greatest number of ancillary reagents and equipment
- Highest number of allele failures and allele dropouts

Work Flow of MIA FORA

Day 1
- Set up long range PCR to run overnight (35 minute set up for 24 samples, 6 hour span time)

Day 2
- Quantify amplicons / Gene balancing
- Library construction / Size selection / Library amplification
- Put on sequencer

Day 3
- After ≈ 24 hours sequencing time, start data processing (can be done remotely, 10 minutes per sample)

Day 4
- Data available first thing in the morning
- Techs analyze
- Report samples by EOB
Advantages of MIA FORA

- Software is very powerful, and has many excellent extra features such as summary window with visual icons, coverage plots, smart guide, IMGT/extended read reference, LD info, etc.
- Good experience with the company — very willing to help, responsive to suggestions, improvements with the FLEX kits, availability
- Easily automated (we have not done this)
- Relatively straightforward protocol for both regular and shortened protocol
- Minimal number of reagents (initial PCR is just master mix and DNA)
- Works well with low concentration samples
- Have not seen any allele dropouts that were not flagged by software

Disadvantages of MIA FORA

- Higher sample volumes
- Limited number of samples depending on equipment, FLEX kit allows for 96 sample but only on Illumina MiniSeq
- Long range PCR not as simple as manufacturer A
- Library construction not quite as easy as manufacturer C
- Requires server and other ancillary equipment

Other Considerations

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>MIA FORA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hands on Time</td>
<td>11.5 hours, 1.5 of this includes 2 techs (option to automate reduces to 8.5, 1.5 with 2 techs)</td>
<td>10.5 hours, 3 hours of this includes 2 techs</td>
<td>5.5 hours, 1.75 of this includes 2 techs</td>
<td>5 hours, 2 hours include 2 techs</td>
</tr>
<tr>
<td>Ancillary Reagents</td>
<td>Qubit, AMPure beads, special plates for bead cleanup</td>
<td>Enzyme, AMPure beads, iDNAquick PCR purification kit, PicoGreen</td>
<td>Long range PCR kit, Promega Quantifluor, KAPA, PippinPrep, AMPure Beads</td>
<td>Qubit, PicoGreen, PippinPrep, special plates</td>
</tr>
<tr>
<td>Ancillary Equipment</td>
<td>Qubit, server, optional (speeds up data processing)</td>
<td>Fluorescent plate reader or Qubit</td>
<td>Fluorescent plate reader</td>
<td>Fluorescent plate reader, PippinPrep, RT instrument</td>
</tr>
</tbody>
</table>
Rankings

<table>
<thead>
<tr>
<th>Rank</th>
<th>Ease of Protocol</th>
<th>Ease of Software</th>
<th>Customer/Tech Support</th>
<th>Reliability of Results</th>
<th>Low Number of Ambiguities</th>
<th>Ease of Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MIA FORA</td>
<td>MIA FORA</td>
<td>MIA FORA, A</td>
<td>MIA FORA, A</td>
<td>MIA FORA, A, B</td>
<td>B</td>
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<tr>
<td>B</td>
<td>MIA FORA</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>B, C</td>
<td>C</td>
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<tr>
<td>C</td>
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<td>B</td>
<td>B</td>
<td></td>
<td>A</td>
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Marianne E. Yee, M.D., MSc
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