Continuing Education Webinar Series

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Future Webinars

12 April Post-Haematopoietic Stem Cell Transplant Chimerism Testing and Engraftment Monitoring

featuring Dr Anil Handoo, Sr. Consultant and Director Pathology BLK Super Specialty Hospital New Delhi, India

Link to register: https://immucor.webinato.com/register
Handouts


Continuing Education

• ABHI, PACE, Florida and California DHS
• 1.0 Contact Hours
• Each attendee must register to receive CE at: https://www.surveymonkey/OptimizeHLAwithImmucor
• Registration deadline is April 13, 2018
• Certificates will be sent via email only to those who have registered by April 27, 2018

Presentation Recording

• Session will be recorded and posted.
  – Access information will be sent to each registrant when the recording becomes available
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Learn website: learn.immucor.com
Questions?

- You are all muted
- Type in questions

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Optimization of HLA Antibody Testing.

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Disclosure

• Nothing significant to disclose

.....still waiting for attractive offers

Single Antigen Bead (SAB)
Luminex assay

• Used by most HLA labs for HLA antibody testing.

• Revolutionized HLA antibody identification and virtual crossmatching.

• Number of advantages compared to Flow SAB and ELISA.
  – ↑ number of analytes tested simultaneously
  – High throughput
  – Rapid analysis

• Still, the procedure is time intensive and is not optimal for use in urgent cases....
  – Friday afternoons come to mind.....

• Some important limitations:
  – Susceptible to interfering substances (“prozone” effect).
Outline

- Optimization of HLA antibody testing
  - **Rapid Optimized Single Antigen Bead (ROB)** protocol for LABScreen (Human Immunology 2017).
    - Development
    - Validation
    - Multicenter evaluation
  - **Enhanced** and **ROB** protocols for LIFECODES LSA.
    - Multicenter evaluation
  - Development of a novel, prozone-resistant **Dual Antibody Rapid Test (DART)** protocol for LABScreen (ASHI Quarterly 2017).

**Single antigen bead (SAB) Luminex LABScreen and LIFECODES LSA protocols**

- Incubate beads (5 μl) and serum 20 μl (RT) 30 min.
- Wash x3 (5 min/spin) 15 min.
  - **Filter plate** 5 min.
- Incubate with 100 μl anti-IgG-PE, 1:100 dilution (RT) 30 min.
- Wash x2 (5min/spin) 10 min.
  - **No wash** 0 min.
- **Total assay time** 1h 25 min.
- Evidence for incubation time/reagent concentration? 1h 5 min.
- Wash times? 2h

**Transfusion Medicine**

- Red cell antibody testing (IAT)
  - How long does it take?
  - **25-30 minutes!!!**
  - Can SAB assay be optimized and expedited?
Objectives

• To develop a rapid single antigen bead LABScreen protocol without compromising the sensitivity of the assay.

• Investigate the effects of:
  – Centrifugation time
  – Serum incubation time
  – Anti-IgG-PE incubation time
  – Serum volume
  – Anti-IgG-PE concentration

Effect of reduced spin time (1 vs 5 min) on bead counts
Effect of reduced spin time

- Standard
  - 5 washes x 5 min = 25 min
  - 1300 x g
- Rapid
  - 5 washes x 1 min = 5 min
  - 1800 x g

No impact on bead counts or overall results
20 minutes saved!

Effects of reduced incubation times

- Serum incubation time
- Anti-IgG-PE incubation time

Effects of reduced incubation time
¼ PPC, HLA class I

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<td>15000</td>
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</tr>
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<td>20000</td>
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3/19/2018
Effects of reduced incubation time
¼ PPC, HLA class I

MFI

Bead number


Effects of reduced incubation time
¼ PPC, HLA class I

MFI

Bead number


Effects of reduced incubation time
¼ PPC, HLA class I

MFI

Bead number

Effects of reduced incubation time
¼ PPC, HLA class II

Effects of reduced incubation time
Negative control serum

Effects of reduced incubation time
NC and PC beads

Significant Effect on IgG binding
Small Effect on background
Conclusion

- Reduction in incubation time with serum and/or anti-IgG-PE results in decreased MFI values.
- Negligible impact on LSNC and NC bead reactivity.
- The degree of MFI decrease when incubation time with anti-IgG-PE was reduced was surprising.
- IgG-PE concentration appears to be sub-optimal?

Effects of increasing IgG-PE concentration ¼ PPC, HLA class I

Effects of increasing IgG-PE concentration ¼ PPC, HLA class II
Conclusion

- Increasing the anti-IgG-PE concentration from 1:100 to 1:5 increases MFI in the standard assay including PC bead MFI.

- Negligible effect on background (LSNC and NC bead).

- Can we compensate for reduced MFI values in the 15/5 min protocol by optimizing the concentration of anti-IgG-PE?
Effects of increasing IgG-PE concentration on MFI in 15/5 protocol
¼ PPC, HLA class I

Effects of increasing IgG-PE concentration on MFI in 15/5 protocol
¼ PPC, HLA class I

Conclusion

• Increasing concentration of anti-IgG-PE compensates for the reduction in incubation times.

• IgG-PE concentration of 1:10 closely matches MFI obtained with the standard assay.

ROB LABScreen® Protocol

- Incubate beads (5 μl) and serum 25 μl (RT) 15 min.
- Wash x3 (1 min/spin) 3 min.
- Incubate with 20 μl anti-IgG-PE, 1:10 dilution (RT) 5 min.
- Wash x2 (5 min/spin) 2 min.
- Total assay time 25 min.


70% time reduction!
Standard vs ROB protocol, MFI correlation
8 patient, 9 ASHI PT, 3 ABH PT sera

Class I

Class II

$y = 1.1803x + 66.752$
$R^2 = 0.9068$

$y = 1.1256x + 92.822$
$R^2 = 0.9845$

Representative Serum Reactivity
Standard vs ROB protocol

AC-463 Class I

AC-463 Class II

“Discrepant” reactions
Cut-off 2000 MFI
1.1 rxn/panel
44 rxn/40 panels
**Conclusion**

- We can reduce the time it takes to perform LABScreen® SAB Luminex assay without compromising assay sensitivity.
- Correlation between the Standard and ROB protocols is excellent.
- No significant impact on test results when using ROB protocol.
- Significant time savings.
- ROB protocol allows for rapid testing of urgent patient sera.
  - Ex. testing during deceased donor work up.

Robert Liwski, Patricia Campbell, Adriana Colosai, Deborah Crowe, Anne Halpin, Ronald Kerman, Dong Li, John Lunz, Cathi Murphey, Peter Nickerson, Denise Pochlewo, Sandra Rosen-Bronson, Olga Timofeeva, Paul Warner, Adriana Zeivi

Participating Centers

- Dalhousie University, Halifax, NS, Canada
- University of Alberta, Edmonton, AB, Canada
- Montefiore-Einstein Transplant Center, Bronx, NY
- Dialysis Clinic Inc. (DCI) Laboratory, Nashville, TN
- Baylor College of Medicine, Houston, TX
- Medstar Georgetown University Hospital, Washington, DC
- University of Pittsburgh Medical Center, Pittsburgh, PA
- Southwest Immunodiagnostics Inc. Laboratory, San Antonio, TX
- University of Manitoba, Winnipeg, MB, Canada
- Puget Sound Blood Center, Seattle, WA

Design

- 2014 ASHI PT sera
  - AC460-464

- Tested by LABScreen SAB Luminex assay
  - Standard lab method
  - ROB protocol
  - Same lot of class I and class II beads

- Result analysis:
  - MFI comparison
  - CV
  - Pearson's correlation ($R^2$)
  - Specificity assignment
  - Pos/Neg ctrl beads (signal vs noise)
AC460 class II
Average lab MFI and CV comparison

Overall mean MFI correlation

Average CV
Standard vs ROB protocol

Serum
Conclusion

- Confirmed excellent correlation between the Standard and ROB protocols.

- Confirmed that there is no significant impact on test results when using ROB protocol.

- ROB protocol appears to improve precision of the results

Liwski et al ASHI 2014
LIFECODES LSA SAB Assay Evaluation

Objectives

- 20 well characterized and challenging sera
- Standard LIFECODES LSA vs ROB protocol

Single Antigen Bead (SAB) Luminex Assay
ROB and Standard LIFECODES LSA protocols

- Incubate beads (10 µl) and serum 25 µl (RT) 40 µl 10 µl 15 min. 30 min.
- Wash x3 (1 min/spin) 3 min. 1 min.
  Filter plate
- Incubate with 20 µl anti-IgG-PE, 1:2 dilution (RT) 50 µl 1:10 5 min. 30 min.
- Wash (1 min/spin) 1 min. 0 min.
  No wash
- Total assay time 25 min.
  1h
Case 5, low titer DSA

Case 5, low titer DSA

Case 7, low titer Aw4/Bw4
Case 7, low titer Aw4/Bw4

Case 2 “prozone” effect, interfering substance

Case 2 “prozone” effect, interfering substance
Summary

- Good correlation between ROB and Standard LIFECODES LSA protocol in many cases.

- ROB protocol exhibits enhanced MFI
  - enhances weak reactivity with low titer DSA.
  - enhances reactivity with low titer abs directed against CREGs.

- Standard LSA protocol is less susceptible to the “prozone” effect compared with the ROB protocol.
  - Treatment with EDTA resolves the “prozone” effect.

- Differences are likely due to serum dilution in the Standard protocol.

Enhanced LIFECODES LSA Protocol

- Immucor developed an enhanced LSA protocol to generate higher MFI values.

- Motivation was based on feedback from the worldwide HLA community.
  - Clinical correlations with MFI have been established.
  - In order to encourage more widespread adoption of the LIFECODES LSA kits, MFI values need to be in line with what clinicians are used to seeing.

- Enhanced LSA protocol uses 20 μl instead of 10 μl of serum per reaction to increase the MFI values.

Participating Centers

- Dalhousie University, Halifax, NS, Canada
- Institut Armand-Frappier, Laval, QC, Canada
- University of Toronto, Toronto, ON, Canada
- Western University, London, ON, Canada
- University of Manitoba, Winnipeg, MB, Canada
- University of Alberta, Edmonton, AB, Canada
- University Of British Columbia, Vancouver, BC, Canada
- Thomas Jefferson University Hospital, Philadelphia, PA
- Johns Hopkins University, Baltimore, MD
- University of Pittsburgh Medical Center, Pittsburgh, PA
- Wake Forest School of Medicine, Winston-Salem, NC
- University of Utah, Salt Lake City, UT
- Southwest Immunodiagnostics Inc., Lab, San Antonio, TX
- Queen Mary Hospital, Hong Kong

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- Southwest Immunodiagnostics Inc., Lab, San Antonio, TX
- Queen Mary Hospital, Hong Kong
Design

- Class I and II HLA LIFECODES LSA kits, filter trays and high speed rotators (provided by Immucor).

- Technical support provided by Immucor (Dusanka D’Atri) to labs not using Immucor kits routinely.

- 9 ASHI PT sera (provided by Immucor) (2014-2016 surveys).
  - AC460, 464, 469, 470, 474, 480-484

Design

- Tested by LIFECODES LSA SAB Luminex assay
  - Standard protocol (40:10)
  - Enhanced protocol (40:20)
  - ROB protocol

- Result analysis:
  - Pos/Neg ctrl beads (signal to noise differential)
  - MFI comparison
  - Mean and SD
  - Pearson’s correlation ($R^2$)
  - Specificity assignment

Negative/Positive Control Beads
Signal to noise differential

- Side by side

- Pos/Neg ctrl beads
- MFI comparison
- Mean and SD
- Pearson’s correlation ($R^2$)
- Specificity assignment
Serum 2 Class II (weak/moderate abs)

Pearson’s correlation, class I HLA

Pearson’s correlation, class II HLA
Pearson’s correlation
Enhanced vs ROB

Class I HLA

Class II HLA

Class I HLA MFI comparison

Comparison of MFI Ranges

Class II HLA MFI comparison

Courtesy Dr. Bryan Ray, Immucor
CV comparison

Class I HLA

Class II HLA

Class I HLA, comparison to ASHI PT Consensus

Class II HLA, comparison to ASHI PT Consensus

Courtesy Dr. Bryan Ray, Immucor
Conclusion

• Enhanced and ROB protocols increase sensitivity (MFI values) in LIFECODES LSA assay (Enhanced > ROB).

• Improve signal to noise differential with no significant impact on background reactivity.

• Good overall correlation between all three protocols (best between Enhanced and ROB).

• Enhanced and ROB protocol show improved concordance of MFI and results.

• ROB protocol confers significant time saving allows for rapid testing of urgent patient sera.
  • Ex. testing during deceased donor work up.


Anna Greenshields, Robert Bray, Howard Gebel and Robert Liwski

Department of Pathology, Dalhousie University
Halifax, Nova Scotia, Canada

Interfering Substances “Prozone” Effect
IgG SAB neat

IgG SAB neat

IgG SAB 1:10

“Prozone” effect
"Prozone" effect

Low titer
Non C fixing Ab

SAB
HLA-A2
“Prozone” effect

Low titer
Non C fixing Ab

“Prozone” effect

High titer
C fixing Ab

“Prozone” effect

High titer
C fixing Ab
“Prozone” effect

High titer
C fixing Ab
C1q binds

C1r & C1s recruited
C4 converted to C4b

C4b

Ca²⁺
“Prozone” effect

High titer C fixing Ab 
C1q binds 
C1r & C1s recruited 
C4 converted to C4b 
C4b binds HLA-Ab complex 

Ca²⁺ 

SAB HLA-A2 

"Prozone" effect

High titer C fixing Ab 
C1q binds 
C1r & C1s recruited 
C4 converted to C4b 
C4b binds HLA-Ab complex 
C2 converted to C2a 

Ca²⁺ 

SAB HLA-A2 

"Prozone" effect

High titer C fixing Ab 
C1q binds 
C1r & C1s recruited 
C4 converted to C4b 
C4b binds HLA-Ab complex 
C2a binds C4b (C3 convertase)
"Prozone" effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b
“Prozone” effect

High titer C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b
C3b binds HLA-Ab complex
and C4b2a (C5 convertase)

Binding of anti-IgG-PE is blocked
HLA antibody not detected

Solutions:
Heat treatment (56°C), destroys C1q and other C
Serum dilution, dilutes out complement
DTT, breaks C1q
EDTA, chelates Ca2+

Objectives

• To develop a SAB protocol that is resistant to the
  “prozone” effect without serum treatment........
Rapid Optimized SAB (ROB) LABScreen® Protocol

- Incubate beads and serum: 15 min.
- Wash x3 (1 min/spin): 3 min.
- Incubate with 20 μl anti-IgG-PE, 1:10 dilution: 5 min.
- Wash x2 (5min/spin): 2 min.
- **Total assay time**: 25 min.

70% time reduction!

Dual Antibody Rapid Test (DART) LABScreen Protocol

- Incubate beads and serum: 15 min.
- Wash x3 (1 min/spin): 3 min.
- Incubate with 20 μl anti-IgG-PE and anti-C'-PE: 5 min.
- Wash x2 (5min/spin): 2 min.
- **Total assay time**: 25 min.

70% time reduction!

Study design

- 20 "prozone" positive sera, 10 class I and 10 class II
  - Tested by SAB:
    - Anti-IgG-PE
    - Anti-IgG-PE with EDTA
    - Anti-C'-PE
    - DART, Anti-IgG-PE + anti-C'-PE
  - Comparison of MFI
Serum 1, Class I HLA

IgG

IgG EDTA

Serum 1, Class I HLA

IgG

IgG EDTA

Serum 1, Class I HLA

IgG

IgG EDTA
## Serum 2, Class II HLA

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<th>IgG</th>
<th>IgG EDTA</th>
<th>C'</th>
<th>IgG/C' DART</th>
</tr>
</thead>
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<td>&lt; 1,000 MFI</td>
<td>No Prozone</td>
<td>Slight</td>
<td>Moderate</td>
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<tr>
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<td>N=739</td>
<td>N=51</td>
<td>N=95</td>
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<tr>
<td>&gt; 1,000 MFI</td>
<td>No Prozone</td>
<td>Slight</td>
<td>Moderate</td>
</tr>
<tr>
<td>N=737</td>
<td>N=739</td>
<td>N=51</td>
<td>N=95</td>
</tr>
</tbody>
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Conclusions

• DART protocol is resistant to the "prozone" effect.

• Eliminates the necessity to treat sera thus avoiding potential interference with HLA antibody testing.

• Improved MFI correlation for "prozone" negative specificities compared with EDTA.

Dual Antibody Rapid Test (DART)

Final thoughts

• Value of protocol optimization
  – Impact on test quality, TAT and clinical patient care
  – Should be an integral part of any assay validation

• Significant variability in antibody testing protocols and results
  – Impact on patient care
  – Impact on transplantation research

• Assay standardization improves concordance of results
Acknowledgements

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  – Geoff Adams
  – Geoff Peladeau
  – Kelly Heinstein

• **Immucor Team**
  – Dr. Bryan Ray
  – Dr. Masako Osada
  – Dusanka D’Atri
  – Kelly Cousins
We like you!
Like us on social media!

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• Type in questions

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featuring
Dr Anil Handoo, Sr. Consultant and Director Pathology
BLK Super Specialty Hospital
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Thank you!