Conclusion
Ravindranath et al have demonstrated that the Immucor LIFECODES Single Antigen Beads contain only the clinically relevant HLA-I trimer, unlike the SAB bead sets from One Lambda’s LABScreen and iBeads, which contain both the HLA-I trimer as well non-clinically relevant structural variants.

The One Lambda LABScreen beads contain non-native HLA-I structural variants, otherwise known as “denatured antigens” which can bind to non-clinically relevant antibodies; therefore using the LABScreen kit for DSQA screening is more likely to produce false positive results. False positives may result in the “inappropriate assignment of unacceptable antigens during transplant listing” which may result in a patient not receiving a transplant even though the donor is a compatible match. Therefore using the Immucor LIFECODES Single Antigen Bead assay which contains exclusively the native HLA-I trimer, HLA laboratories can avoid unnecessary false positivity and potentially facilitate more patients receiving an organ transplant.

The authors conclude that:
- There is significant MFI inter- and intra-assay variability when using Single Antigen assays from both One Lambda and Immucor.
- On average, MFI values using the LABScreen Single Antigen assay were higher than those obtained using the LIFECODES assay.
- Higher MFIs when using LABScreen beads is not unexpected because they contain both native and denatured antigens.
- One Lambda’s LABScreen resulted in more samples with HLA-I antigens deemed unacceptable.
- The presence of denatured antigen on the LABScreen beads leads to false positive HLA-I results.
- The presence of denatured antigen on the LABScreen beads will also lead to false positive complement testing results (C1q and C3d).
- It is important to consider the higher reactivity and false positivity of the LABScreen assay when choosing a Single Antigen vendor to avoid denying patients “otherwise acceptable organs” or initiating “inappropriate desensitization procedures”.

References
Sullivan et al., Understanding solid-phase HLA antibody assays and the value of MFI. Human Immunology 78 (2017) 471-480.
Hilton H.G. and Parham P., Direct binding to antigen-coated beads refines the specificity and cross-reactivity of four monoclonal antibodies that recognize polymorphic epitopes of HLA class I molecules. Tissue Antigens 81, 2013, 212-220.
LIFECODES® Single Antigen Assay: Lowering the Denatured Antigen Barrier

Introduction
While Single Antigen Testing becomes an increasingly important test to determine the presence of unacceptable antigens pre-transplant and to determine the necessity of desensitization treatment post-transplant, false positive reactivity due to the presence of denatured HLA antigens on the beads presents a well-characterized clinical challenge. There are four possible forms of HLA Class I (HLA-I) structural variants that can be present on Single Antigen Beads. The “native” HLA-I trimer (Figure 1) is found within natural cells whereas the other three structural variants, otherwise described as “denatured antigens” (Figure 2), are by-products of the Single Antigen Bead manufacturing process.

Of these four variants, only the native HLA-I trimer has been associated with a significantly lower graft survival rate. Non-native HLA-I structural variants contain altered epitopes that can react with non-clinically relevant HLA-I antibodies and cause false positive reactivity. When Single Antigen assays indicate Donor Specific Antibody (DSA) is present and the crossmatch is negative, many labs spend considerable resources trying to understand if the Single Antigen results are clinically relevant.

In an abstract recently published by the Cleveland Clinic in Human Immunology, Zhang et al demonstrate that 85% of false positives in their laboratory are due to denatured antigens. Multiple analyses are required to identify false positives due to denatured antigen including epitope analysis, reviewing specific antibody testing patterns, intensities and consistency from sample to sample. To resolve the false positives, they take many follow up steps including re-testing on screening and phenotype beads, acid treatment, comparing the results to the patient history and performing surrogate cross matches.

Figure 1. Native HLA trimer consists of an Alpha or “Heavy” Chain, Beta 2 microglobulin, and a peptide.

If not clearly identified and resolved, false positive results may result in the “inappropriate assignment of unacceptable antigens during transplant listing” which may consequently result in a transplant even though the donor is a compatible match. Additionally, DSA monitoring is routinely performed to determine if immunosuppression should be increased or decreased post-transplant. False positives could result in treating patients with immunosuppressive therapy unnecessarily.

A variety of studies have been performed to characterize the antigen composition on Single Antigen Beads (SAB) available from One Lambda and Immucor. These studies demonstrate how the antigen composition on the beads contributes to false positive results when running Single Antigen assays.

In 2017, Ravindranath et al demonstrated the Immucor LIFECODES SAB bead sets carry exclusively the HLA-I trimer whereas the SAB bead sets from One Lambda, the LABScreen® and Beads (discontinued), carry both the native HLA-I trimer as well as one or more non-native structural variants. Because the structural variants can interact with non-clinically relevant antibodies, they are more likely to cause false positive reactivity. To more accurately quantify the captured antibody density, Ravindranath et al also tested the samples using a monoclonal detection antibody specific for the Free IgH/PolyFab. Using the same protocol and detection method ensured that the difference in LABScreen and LIFECODES results were due specifically to bead antigen composition.

Because IgH/PolyFab is polyclonal, it can bind to and detect antibodies captured by bead bound HLA antigens multiple times depending on the antibody stoichiometry. This may cause an over assessment of antibody density bound to the beads. To more accurately quantify the captured antibody density, Ravindranath et al also tested the samples using a monoclonal detection antibody specific for the Free Chain on the native HLA antigen: PE-conjugated Mouse FC-specific Anti-Human IgG (Southern Biotech). The monoclonal detection antibody is referred to in this publication as IgHPolyFab. The IgHPolyFab was titrated to 10 ug/mL, and the MFI cutoff of 1,000 was used to determine positive results due to mAB binding to the antigens on the beads.

For this study, the samples were tested using the One Lambda protocol for both assays (using 2uL of beads rather than 5uL), and the One Lambda detection antibody: PE-conjugated Goat Anti-human IgG (Canoga Park, CA, USA) binds to β2aHC (pepA-β2aHC and pepF-β2aHC).

Methods
In the 2018 study, Ravindranath et al confirmed the results of their 2017 publication using samples from End Stage Renal Disease (ESRD) patients that had either been accepted for or were rejected for a kidney and/or liver transplant. Although the patients were accepted for transplant, these samples were suspected to have had produced false positive results in previous Single Antigen testing.

For this study, the samples were tested using the One Lambda protocol for both assays (using 2uL of beads rather than 5uL) and the One Lambda detection antibody: PE-conjugated Goat Anti-human IgG (Catalog # LS-ASB2). The One Lambda detection antibody is polyclonal and referred to in the publication as IgHPolyFab. Using the same protocol and detection method ensured that the difference in LABScreen and LIFECODES results were due specifically to bead antigen composition.

Because IgHPolyFab is polyclonal, it can bind to and detect antibodies captured by bead bound HLA antigens multiple times depending on the antibody stoichiometry. This may cause an over assessment of antibody density bound to the beads. To more accurately quantify the captured antibody density, Ravindranath et al also tested the samples using a monoclonal detection antibody specific for the Free Chain on the native HLA antigen: PE-conjugated Mouse FC-specific Anti-Human IgG (Southern Biotech).

Results
The native HLA-I trimer is comprised of two chains and a peptide: alpha chain, beta 2 microglobulin (β2M) chain and a peptide. The native HLA-I trimer has a specific epitope that binds to clinically significant HLA-I DSA. Patients with DSA to the native HLA-I trimer have been shown to have a significantly lower graft survival rate when compared to patients with no DSA or antibodies to non-native HLA-I structural variants.

When HLA-I recombinant proteins are manufactured for SAB assays, any combination of the two amino acid chains and peptide can result. The non-native HLA-I structural variants assume epitopes that react with non-clinically relevant antibodies and can lead to false positive reactivity.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Specificity - Positive</th>
<th>Specificity - Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6/32 IgHPolyFab</td>
<td>pepA-β2aHC and pepF-β2aHC</td>
<td>β2M</td>
</tr>
<tr>
<td>TFL-006 IgHPolyFab</td>
<td>pepE β2aHC and β2aHC</td>
<td>β2aHC</td>
</tr>
</tbody>
</table>

Figure 3. Monoclonal antibody specificities to the HLA-I structural variants surveyed by Ravindranath et al.
LIFECODES® Single Antigen Assay: Lowering the Denatured Antigen Barrier

In the 2017 study, Ravindranath et al used three monoclonal antibodies (mAb’s) that react with different HLA-I antigen structural variances to detect which HLA-I variants were present on three Single Antigen Bead sets: LABScreen (One Lambda), iBeads (One Lambda) and LIFECODES Single Antigen Assay (Immucor).

Figure 7 summarizes the median MFI of each SAB assay when hybridized with mAb HC-10. mAb HC-10 binds to the peptide free HLA-I structural variants: Alpha Chain Only (B2fHC) and Alpha Chain + B2M Dimer (pep-F-B2aHC). Although there is some mAb HC-10 signal with the Immucor LIFECODES beads, the signal is lower than the established positive threshold of <1,000. The One Lambda iBeads signal indicates a presence of the HLA-I structural variants on the beads with signal high enough to be called positive using the 1,000 MFI cutoff.

In contrast, the very high signal on the One Lambda LABScreen beads indicates a very large amount of Alpha Chain only and Alpha Chain + B2M dimer presence on the beads. Ravindranath et al suspect the high reactivity of the One Lambda LABScreen beads with mAb W6/32 is due to the presence of the Alpha Chain + B2M Dimer and not purely to an increased density of the native HLA-I trimer on the beads as was suggested by Hiton and Parham.

Figure 8 summarizes the median MFI of each SAB assay when hybridized with mAb W6/32. The positive reaction with mAb W6/32 across all loci shows all three SAB assays contain the clinically relevant native HLA-I trimer. The stronger signal of the LABScreen and iBeads is due to the mAb W6/32 reaction with the structural variant that does not include the peptide as demonstrated by their reactivity with mAb HC-10 outlined below.

Figure 5. Monoclonal antibody reactivity patterns indicative of the presence of each HLA-I structural variant.

mAb W6/32

Figure 6 summarizes the median MFI of each SAB assay when hybridized with mAb W6/32. The positive reaction with mAb W6/32 across all loci shows all three SAB assays contain the clinically relevant native HLA-I trimer. The stronger signal of the LABScreen and iBeads is due to the mAb W6/32 reaction with the structural variant that does not include the peptide as demonstrated by their reactivity with mAb HC-10 outlined below.

Figure 6. Representation of the data generated by Ravindranath et al demonstrating that all three bead sets contain the native HLA-I trimer. The higher signals with the One Lambda LABScreen beads may be due to the presence of the peptide free structural variant.

Figure 7. Representation of the data generated by Ravindranath et al demonstrating that only the Immucor LIFECODES beads are negative (<1,000 MFI) for two HLA-I non-clinically relevant structural variants. The One Lambda iBeads demonstrate low positivity, while the One Lambda LABScreen beads show very high positivity for the clinically non-relevant HLA-I structural variants.

mAb HC-10
Based on the mAb binding patterns, Ravindranath et al concluded that the One Lambda LABScreen beads contain both the native HLA-I trimer and all three structural variants, the One Lambda iBeads (discontinued) contained the native HLA-I trimer along with both the peptide free and peptide associated B2fHC structural variants, whereas only the Immucor LIFECODES Single Antigen beads carry the native HLA-I trimer “exclusively” and are therefore more useful for monitoring of the clinically relevant HLA-I DSAs.

In the 2018 study, Ravindranath et al tested 30 samples from ESRD patients that had either been accepted for transplant or received transplants. The testing was done with both One Lambda LABScreen and Immucor LIFECODES Single Antigen assays per the protocol outlined in the Methods section.

50% (15) of the samples were HLA-I negative with both vendors’ assays when using the polyclonal detection antibody IgHPolyFab and the monoclonal detection antibody FcMonoIgG. 30% (9) of the samples were HLA-I positive with results consistent between both vendors’ assays. These samples are likely from post-transplant patients that have developed DSAs.

17% (5) of the samples were positive with One Lambda’s assay and negative with the LIFECODES assay when using the polyclonal detection antibody IgHPolyFab. When these samples were retested with the monoclonal detection antibody FcMonoIgG, the LABScreen positive result was greatly lowered or eliminated. This indicates the positivity was likely due to denatured antigen on the LABScreen beads. One additional sample demonstrated a decrease in positivity when FcMonoIgG was used to detect the LABScreen result, but resulted in one unacceptable antigen not previously detected in the LIFECODES result. The cause for this variation is unknown.

Figure 8 shows the reactivity of the Single Antigen beads with mAb TFL-0006 which binds to the Alpha chain only when beta 2 microglobulin is absent (B2fHC). mAb TFL-0006 reactivity is indicative of either the Alpha Chain with the Peptide or the Alpha Chain without the Peptide. Strong reactivity was observed with One Lambda’s LABScreen and low reactivity was observed with One Lambda’s iBeads. The LIFECODES Single Antigen beads showed no reactivity with mAb TFL-006 indicating neither of the B2fHC variants are present on the beads.

Figure 9 shows the reactivity of the LIFECODES Single Antigen beads with mAb TFL-006 which binds to the Alpha chain only when beta 2 microglobulin is absent (B2fHC). mAb TFL-0006 reactivity is indicative of either the Alpha Chain with the Peptide or the Alpha Chain without the Peptide. Strong reactivity was observed with One Lambda’s LABScreen and low reactivity was observed with One Lambda’s iBeads. The LIFECODES Single Antigen beads showed no reactivity with mAb TFL-006 indicating neither of the B2fHC variants are present on the beads.