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It's about time: The development and validation of a rapid optimized single antigen bead (ROB) assay protocol for LABScreen

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ABSTRACT

The LABScreen single antigen bead assay (SAB) is a method widely used for the identification and monitoring of human leukocyte antigen (HLA) antibodies in patients pre- and post-transplant. While accurate testing of patient samples is key for optimal patient care, time can also be important, especially during deceased donor workups or post-transplant assessments. Here we describe the development and validation of the **Rapid Optimized SAB (ROB)** protocol, a modified version of the One Lambda LABScreen SAB (OLSAB) procedure, which reduces assay time from 85 to 25 min (>70% reduction) without impacting assay quality or sensitivity. Optimization steps included shortened centrifugation cycles and reduced serum and secondary antibody incubation times in combination with increased secondary antibody concentration. Linear regression analysis of baseline median fluorescence intensity (MFI) values showed excellent correlation between the ROB and OLSAB protocols ($r^2 > 0.98$) for both class I and class II antibodies in 58 sera tested in two HLA laboratories. Importantly, the ROB protocol demonstrated a trend towards improved inter-laboratory MFI concordance when compared to the OLSAB procedure ($r^2 = 0.9816$ vs 0.9451), especially for HLA antibody specificities in the 500–2000 MFI range ($r^2 = 0.7824$ vs 0.6313). Implementation of the ROB protocol will expedite HLA antibody testing and may improve reproducibility of the SAB assay.

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1. Introduction

The presence of pre-formed donor specific HLA antibodies (DSA) can significantly impact post-transplant outcomes [1,2]. Sensitization events such as pregnancy, blood transfusions and/or prior transplants can result in the formation of HLA antibodies, and detection and documentation of these antibodies prior to donor selection aids in the identification of compatible transplant donors

and minimizes the risk of antibody mediated graft rejection [1]. The LABScreen single antigen bead (SAB) luminex assay [3] is routinely used by the majority of histocompatibility laboratories in North America to detect antibodies directed towards HLA antigens as part of pre-transplant patient workup and post-transplant DSA monitoring [4,5]. In this assay, fluorescent polystyrene microbeads, each coated with a single recombinant HLA allele, are incubated with recipient serum to allow HLA-specific antibodies to bind bead-bound HLA antigens. Bound HLA-specific antibodies are then detected with a PE-conjugated anti-human IgG secondary antibody and the corresponding mean fluorescence intensity (MFI) is measured on the Luminex instrument [3]. Since its introduction over a decade ago, the SAB assay has revolutionized histocompatibility testing providing more accurate assignment of HLA antibody specificity [4–6], and improved assessment of immunological risk [5–8] and crossmatch prediction [7,8]. In turn, deceased donor organ allocation [9,10], and living donor kidney paired exchanges [11,12] were positively impacted as compatible donors could be identified by virtual crossmatch, improving the accuracy and speed

Abbreviations: ABH, Brazilian Association for Histocompatibility; ASHI, American Society for Histocompatibility and Immunogenetics; DSA, donor specific HLA antibodies; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; LSNC, LABScreen negative control sera; LWB, LABScreen wash buffer; MFI, mean fluorescence intensity; NC, negative control bead OLSAB One Lambda LABScreen SAB; PC, positive control bead; PE, phycoerythrin; PPS, pooled positive control sera; PT, proficiency testing; ROB, rapid optimized SAB assay; RT, room temperature; SAB, single antigen bead; SWID, Southwest Immunodiagnostics Inc.

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of compatible donor and recipient matching and decreasing the likelihood of a positive crossmatch prior to transplantation. SAB testing has proven to be a key method for the accurate assignment of antibody specificities. Nevertheless, several assay limitations exist and must be taken into account when interpreting results [reviewed in 13,14]. For example, false positive reactions have been linked to the presence of denatured HLA molecules on the SABs [15–17]. In addition, complement mediated interference can lead to false negative reactions, a so called “prozone” effect [18–22], thus necessitating serum treatment with modalities such as dialysis [18], heat [20], dithiotreitol (DTT) [18,20] or ethylenediaminetetraacetic acid (EDTA) [20] in order to minimize this phenomenon. Finally, the SAB assay exhibits substantial intra and inter laboratory result variability attributed in part to differences in protocols used by individual laboratories highlighting the need for assay optimization and standardization [23].

The LABScreen assay uses the Lambda Array Beads Multi-Analyte System [3], which permits relatively rapid testing and analysis of multiple patient samples compared to the FlowPRA® SAB assay or ELISA based testing. Nonetheless, the LABScreen assay is time intensive, taking at least 2–3 h to perform depending on the number of samples tested. High quality and timely testing of serum samples is needed to provide patients with the best possible care. Rapid turnaround times are especially important in the context of deceased donor workups and the 2–3 h required to perform the traditional SAB assay can use up precious time in these urgent cases.

Our group recently developed and implemented a rapid optimized flow cytometry crossmatch assay [24], called the Halifax protocol, which not only can be performed in less than 40 min (a 60% decrease compared to standard flow cytometry crossmatch protocol) [25] but also exhibits improved assay sensitivity compared to standard flow crossmatch methods [26,27]. The data presented herein documents that similar optimization can be achieved for the LABScreen SAB assay.

2. Materials and methods

2.1. Reagents and sera

SAB assays were performed using LABScreen SAB kits (LS1A04 lot 8 for HLA class I and LS2A01 lot 10 for HLA class II; One Lambda, Canoga Park, CA), 100X phycoerythrin (PE)-conjugated goat anti-human IgG (LS-AB2, Lot B53; One Lambda) and 96-well V-bottom trays (Whatman Piscataway, NJ). All washes were performed with LABScreen wash buffer (LWB; One Lambda).

Pooled positive control sera (PPS; a pool of 20 highly sensitized patient sera, diluted 1:4 in PBS) and LABScreen negative control sera (LSNC, lot 13; One Lambda) were used in all SAB assay protocol optimization experiments. A panel of serum samples from two laboratories were used for the ROB protocol validation: (1) Halifax laboratory (n = 36) used sera from patients awaiting transplant (n = 10), American Society for Histocompatibility and Immunogenetics (ASHI) proficiency testing (PT) surveys (n = 20; AC450 – AC464 and AC470–474) and Brazilian Association for Histocompatibility (ABH) PT surveys (n = 6; sera #52–57); (2) Southwest Immunodiagnosics Inc. (SWID) laboratory (n = 22/20 class I/II) used sera from patients awaiting transplant (n = 17/15 class I/II) and ASHI PT surveys (n = 10, AC460–AC464 and AC470–474). All sera were treated with EDTA disodium salt solution at a final concentration of 6.3 mM (0.5 M stock solution, Cat# E7889; Sigma-Aldrich, St Louis, MO), before SAB testing [20].

2.2. Standard One Lambda LABScreen SAB (OLSAB) protocol

The following standard One Lambda LABScreen SAB (OLSAB) protocol (Table 1) was used as the baseline in all optimization

experiments and protocol validation. EDTA-treated sera (20 µl/test) were added to the wells of a 96-well v-bottom tray containing 2.5 µl of either HLA class I or class II SABs (5 µl of SABs were used in some experiments as indicated in Section 3.4). The trays were then incubated for 30 min at room temperature (RT) in the dark on a tray shaker. Trays were washed 3 times with LWB (200 µl/well) using 5 min centrifugation cycles at 1800×g. Next, 100 µl of the working anti-human IgG-PE secondary antibody reagent (1:100 dilution in LWB) was added to each well. Trays were incubated for 30 min at RT, and then washed twice as described above. SABs were then resuspended in 80 µl of LWB and acquired using the Luminex 200 analyzer (Luminex). Results were analyzed using Fusion 3.0 software (One Lambda). Data output included the raw trimmed MFI as well as baseline MFI where background MFI of the negative control (NC) bead and LSNC serum were subtracted. Positive control (PC) bead MFI values were also analyzed.

2.3. OLSAB assay optimization

2.3.1. Centrifugation time

As the first step in assay optimization, plate centrifugation time was shortened to 1 min and compared to the standard 5 min centrifugation. LABScreen HLA class I and class II SABs (1.25 µl/well) were added to 2 separate 96-well v-bottom trays. Both trays were then washed 5 times in LWB, with centrifugation at 1800×g for 1 or 5 min at RT. After the final wash, beads were resuspended in 80 µl of PBS and acquired on the Luminex 200 analyser. The acquisition time for each sample was kept constant at 90 s to detect any change in bead number resulting from the decrease in centrifugation time.

2.3.2. Serum/beads and secondary anti-IgG-PE antibody incubation times

PPS and LSNC sera were used to assess the impact of shortened incubation times on specific and background SAB reactions. Different incubation times were tested for serum antibody binding to beads (30 min vs 15 min) and secondary anti-human IgG-PE antibody binding (30 min vs 5 min). MFI values of HLA SABs as well as the NC and PC beads were determined following acquisition on the Luminex 200 and were subsequently compared among all test conditions.

2.3.3. Anti-human IgG-PE concentration

PPS and LSNC sera were used to determine the optimal concentration of secondary anti-human IgG-PE antibody for the OLSAB and shortened incubation SAB assay protocols. Anti-human IgG-PE antibody dilutions ranging from 1:5 to 1:100 were tested.

Table 1
Comparison of LABScreen SAB protocols.

Parameter	One Lambda LABScreen SAB (OLSAB)	Rapid Optimized SAB (ROB)
EDTA treatment	Yes	Yes
Serum volume	20 µl	25 µl
SAB volume	2.5–5 µL	2.5–5 µL
First incubation time	30 min	15 min
Washes (1st set)	3 × 5 min at 1800×g	3 × 1 min at 1800×g
anti-IgG PE concentration	1:100, 100 µl/test	1:10, 20 µl/test
Second incubation time	30 min	5 min
Washes (2nd set)	2 × 5 min at 1800×g	2 × 1 min 1800×g
Final suspension volume	80 µl	55 µl
Total assay time	85 min	25 min

2.4. Rapid optimized SAB (ROB) protocol testing and validation

The ROB protocol (Table 1), was validated in two separate HLA laboratories by parallel testing of a panel of 58 sera (see Section 2.1 above for details) against the standard OLSAB protocol. Both protocols were tested using 2.5 μ l of either HLA class I or class II SABs per reaction (5 μ l of SABs were used in some experiments as indicated in Section 3.4). The ROB protocol features the following changes compared to the OLSAB assay: (1) all centrifugation times were decreased from 5 to 1 min at a speed of 1800 $\times$ g, (2) serum incubation with SABs was shortened from 30 to 15 min, (3) anti-human IgG-PE secondary antibody staining time was decreased from 30 to 5 min, (4) anti-human IgG-PE working concentration was increased from a 1:100 dilution to 1:10, (5) the anti-human IgG-PE working solution is added at 20 μ l/well rather than 100 μ l/well, and (6) the final bead suspension volume was reduced from 80 μ l to 55 μ l.

2.5. Inter-laboratory comparison of OLSAB and ROB protocol results

ASHI PT samples (AC460–AC464) were tested in parallel using the OLSAB and ROB protocols in both the Halifax and SWID laboratories. Identical lots of the LABScreen SAB kits and LSNC (see Section 2.1) were used in both laboratories. Goat anti-human IgG-PE lot B53 and Lot B54 were used in the SWID and Halifax labs, respectively. Results for each protocol were analyzed and compared between laboratories.

2.6. Statistical analysis

Linear regression analysis was performed using Microsoft Excel software.

3. Results

3.1. Effect of centrifugation time on SAB assay bead counts

The standard OLSAB assay protocol (Table 1) includes 5 wash steps, each involving a 5 min centrifugation cycle, adding 25 min to the assay and accounting for approximately 30% of the total assay time. As a first step in assay optimization, we assessed the impact of shortened centrifugation cycles (from 5 min to 1 min/spin, at 1800 $\times$ g) on the bead acquisition rate and final bead counts following acquisition on the Luminex 200. Interestingly, reduction of centrifugation time had no impact on the event acquisition rate (data not shown). In addition, there was no impact on the final bead counts (Fig. 1). Similar results were seen when One Lambda recommended centrifugation speed of 1300 $\times$ g was used (data not shown). These results indicate that washes with 1 min centrifugations could be used to shorten the assay by 20 min without any impact on these aspects of assay performance. One min centrifugation cycles were therefore used during washes in all subsequent experiments.

3.2. Effect of varying incubation times on the SAB assay

The standard OLSAB assay protocol includes two 30 min incubation steps (Table 1). During the first step, patient sera are incubated with antigen-coated beads to allow HLA-specific antibodies to bind to their target HLA antigens. In the second incubation step, goat anti-human IgG-PE secondary antibody is used to detect the IgG antibodies bound to bead-bound HLA antigens. The data output from the SAB assay includes raw trimmed MFI values and baseline MFI values (with subtracted background NC bead MFI and LSNC serum MFI). While baseline MFI cutoffs defining positive reactions may vary among labs, values of 1000–2000 MFI are typically

employed. A PC bead, coated with human IgG, is used to assess reactivity with the anti-human IgG-PE secondary antibody. Labs frequently use an arbitrary PC bead MFI cutoff or a PC/NC MFI ratio to determine acceptability of the test results. Each of the above parameters was considered during assay optimization.

To assess the impact of shortened incubation times on specific and background SAB reactions, we used PPS and LSNC serum. PPS serum was diluted 1:4 in PBS to avoid saturating bead-bound HLA antigens with high titer HLA antibodies. The LSNC sera were tested in an undiluted form. In order to determine the critical nature of the length of each incubation step (serum antibody binding to HLA antigens on beads and secondary anti-IgG-PE antibody binding), different incubation times were assessed individually as well as in combination. The specific incubation times were selected based on preliminary time course experiments (data not shown) and our previous studies to optimize the flow cytometry crossmatch assay [24]. Reduction of the serum incubation step from 30 to 15 min (15/30 min) resulted in an overall decrease in PPS raw MFI values for both HLA class I (Fig. 2a) and class II (Fig. 2b) beads compared to the OLSAB (30/30 min) protocol. Similarly, the PPS raw MFI values were reduced compared with the OLSAB (30/30 min) protocol when incubation with the secondary antibody was shortened from 30 to 5 min (30/5 min) (Fig. 2a and b). Not surprisingly, when both shortened incubations were combined (15/5 min), there was an additive decrease in the PPS raw MFI values in comparison to the OLSAB (30/30 min) protocol (Fig. 2a and b).

The LSNC background MFI values (Fig. 2c and d) and the NC bead background MFI values (Fig. 2e) were also reduced with shortened incubations. However, the background MFI differences were relatively minor and did not significantly impact baseline MFI values.

Finally, we assessed the effects of shorter antibody incubations on reactivity with the PC bead. As anticipated, reduction of the serum incubation from 30 to 15 min (15/30 min) had no impact on the PC MFI values (Fig. 2f). However, when incubation time with the secondary antibody was shortened from 30 to 5 min (30/5 min and 15/5 min), a marked decrease in MFI (45–50%) was observed. This degree of PC MFI decrease seen when the secondary antibody incubation was shortened suggested that the anti-IgG-PE antibody concentration in the OLSAB protocol (1:100 dilution) was suboptimal.

3.3. Effects of anti-human IgG-PE concentration on MFI values in the SAB assay

To further investigate the impact of secondary antibody concentration on resulting MFI values, different concentrations of anti-human IgG-PE secondary antibody were tested using the OLSAB protocol. Fig. 3 shows that the PPS raw MFI values with the OLSAB protocol for HLA class I (Fig. 3a) and class II (Fig. 3b) beads were enhanced as the concentration of secondary antibody was increased from a dilution of 1:100 to 1:5. Importantly, only minor increases in the LSNC background MFI values (Fig. 3c and d) and the NC bead background MFI values (Fig. 3e) were observed. Finally, PC bead MFI values were markedly enhanced as the concentration of anti-human IgG-PE antibody was increased (Fig. 3f). Collectively, these data demonstrate that the recommended secondary antibody concentration described in the OLSAB protocol is suboptimal and that the signal to noise differential (baseline MFI value) can be improved by increasing anti-human IgG-PE antibody concentration.

Seeing the impact of increased anti-human IgG-PE secondary antibody concentration on MFI values in the OLSAB protocol, we reasoned that increasing the secondary antibody concentration would have a similar effect on MFI with the shortened incubation protocol. To investigate this, we performed parallel experiments, using the

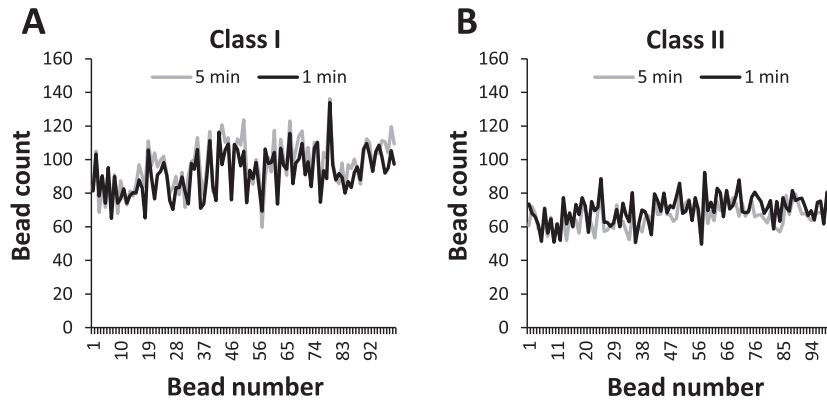


Fig. 1. Shortened centrifugation time during washing has no impact on final bead counts. HLA class I (A) and class II (B) beads (1.25 μ l/well) were washed 5 times (at 1800 $\times$ g) using either 5 min (grey) or 1 min (black) centrifugation cycles. Mean bead counts of 3 independent experiments are shown.

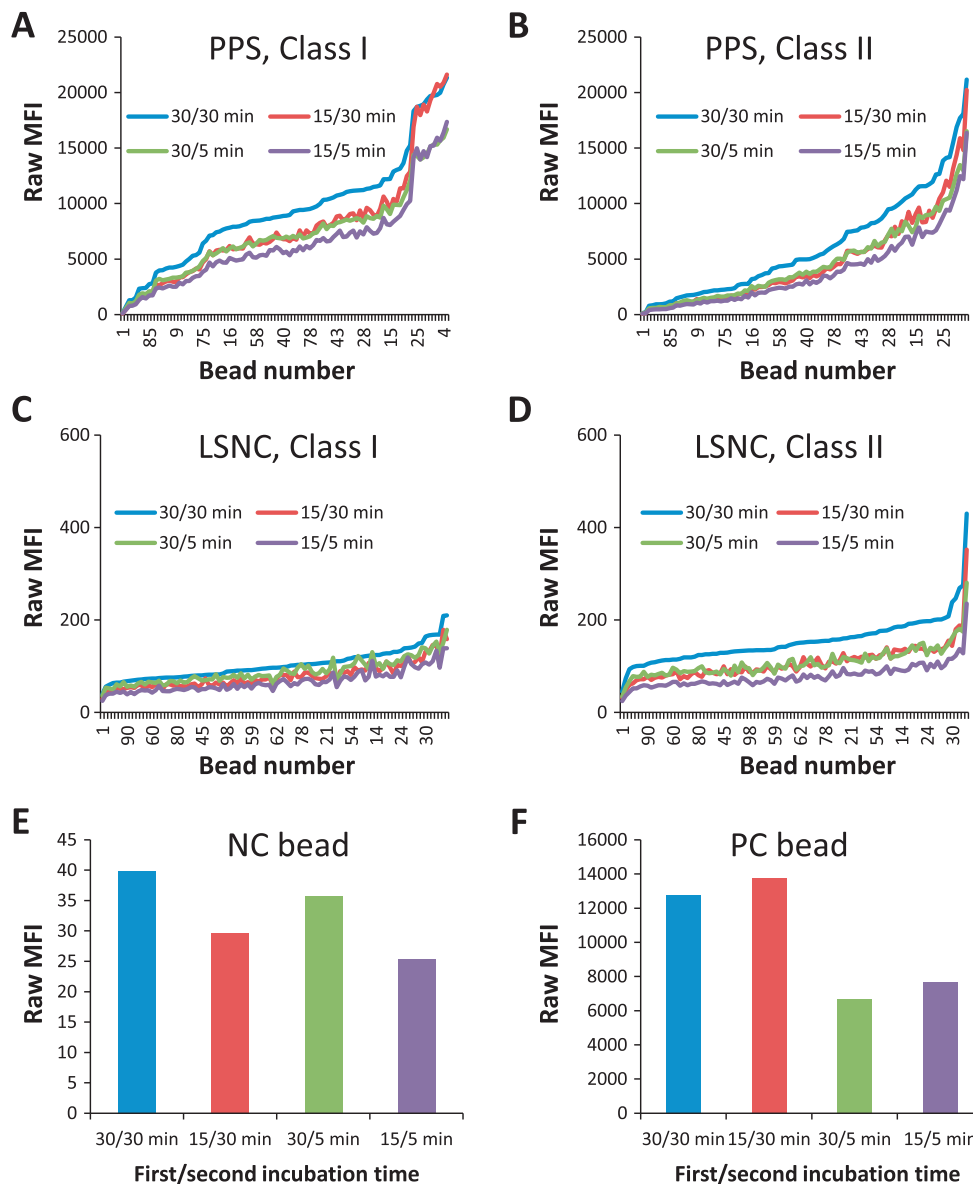


Fig. 2. Shortened incubation times reduce SAB assay MFI. PPS (A, B) and LSN C (C, D) sera were tested by HLA class I (A, C) or class II (B, D) SAB assay using the OLSAB incubation protocol (30/30, blue) (first/second incubation time) or various short incubation protocols where either the first (15/30, red), the second (30/5, green) or both (15/5, purple) incubation times were reduced. NC (E) and PC bead (F) show raw MFI values from LSN C serum HLA class I testing. Representative raw MFI values from 1 of 3 independent experiments are shown.

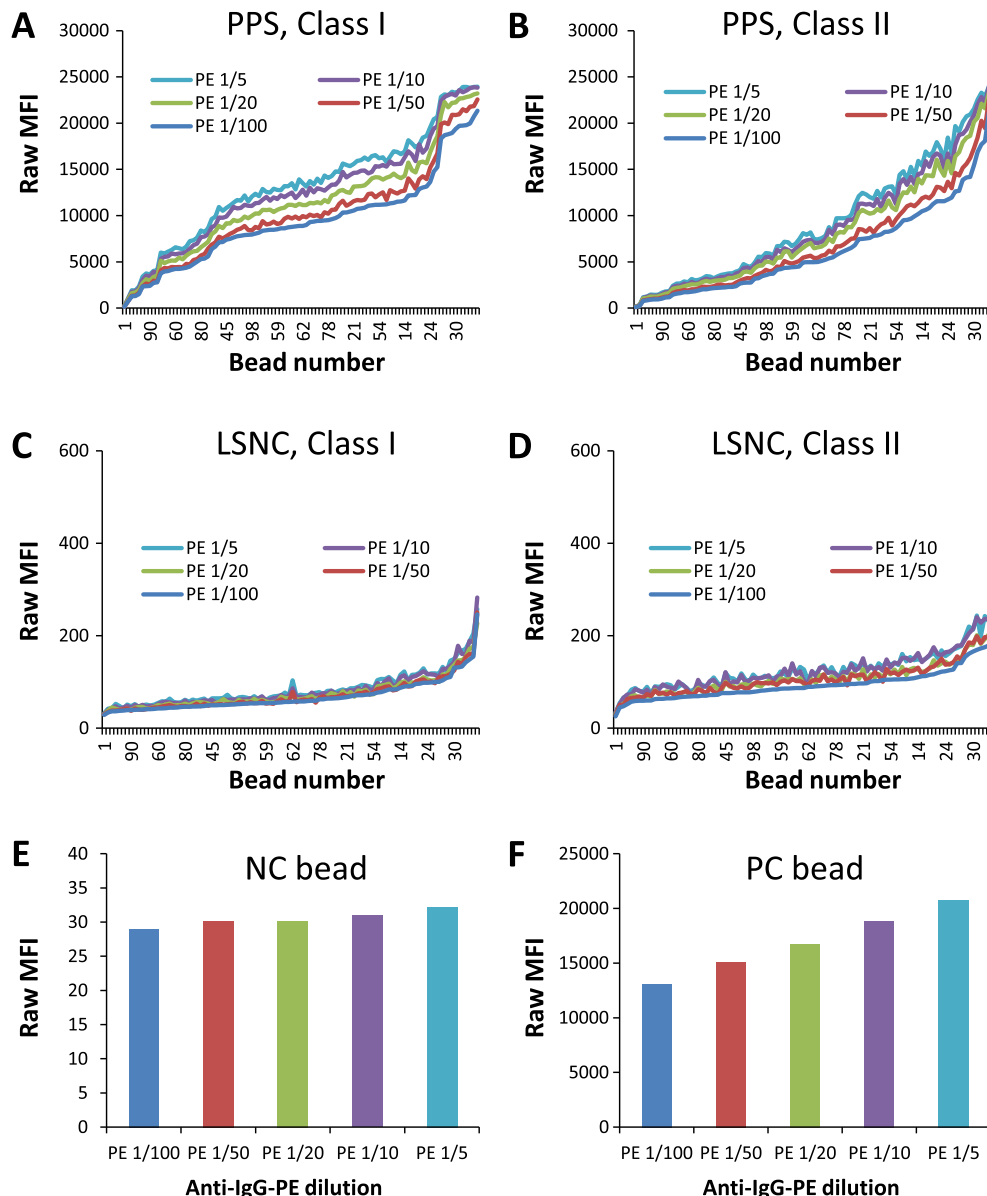


Fig. 3. Increased anti-IgG-PE concentration enhances SAB assay MFI. PPS (A, B) and LSNC (C, D) sera were tested by the HLA class I (A,C) or class II (B,D) OLSAB assay protocol using various dilutions of anti-IgG-PE ranging from 1:100 to 1:5. NC (E) and PC bead (F) show raw MFI values from LSNC serum HLA class I testing. Representative raw MFI values from 1 of 3 independent experiments are shown.

PPS and LSNC sera, comparing the OLSAB (30/30 min) protocol with the standard anti-human IgG-PE dilution (1:100) to the shortened incubation (15/5 min) protocol at various secondary antibody concentrations. As shown in Fig. 4, increased anti-human IgG-PE antibody concentration in the shortened incubation (15/5 min) protocol compensated for reduced PPS raw MFI values observed with the OLSAB protocol. The effects of increased anti-human IgG-PE antibody concentration on background LSNC and NC MFI were negligible (data not shown). Finally, the OLSAB (30/30 min) protocol raw PPS MFI values were best matched when the short incubation (15/5 min) protocol was performed using the 1:10 anti-human IgG-PE antibody dilution (Fig. 4a and b; blue line vs red line).

3.4. Validation of the rapid optimized single antigen bead (ROB) assay using HLA alloantibody containing sera

Based on the results described above (Sections 3.1–3.3), the Rapid Optimized single antigen Bead (ROB) protocol was designed

(Table 1). The protocol features shortened serum and secondary antibody incubation times (15/5 min), reduced centrifugation cycles (1 min/spin), an increased anti-human IgG-PE concentration (1:10 stock dilution, 20 μ l/test) and a reduction of final bead suspension volume from 80 μ l to 55 μ l. To validate the ROB protocol, a panel of well characterized sera were tested in parallel with the OLSAB protocol in two HLA laboratories (Halifax lab: 10 patient, 15 ASHI PT and 6 ABH PT sera; SWID lab: 17/15 class I/II patient and 5 ASHI PT sera). Of the 62 SAB assay panels performed at the Halifax lab (31 HLA class I and 31 HLA class II) by the OLSAB protocol, 40 were HLA antibody positive (20 class I and 20 class II) and 22 were HLA antibody negative (11 class I and 11 class II). When tested with the ROB protocol, all 22 HLA antibody negative panels were negative indicating that the ROB protocol did not result in false positive reactions. Furthermore, the reactivity patterns seen in all 40 HLA antibody positive panels tested with the OLSAB vs ROB protocols were virtually identical. A graph of reactivity with the AC463 (ASHI PT) serum is shown as an example (Fig. 5). Similar results

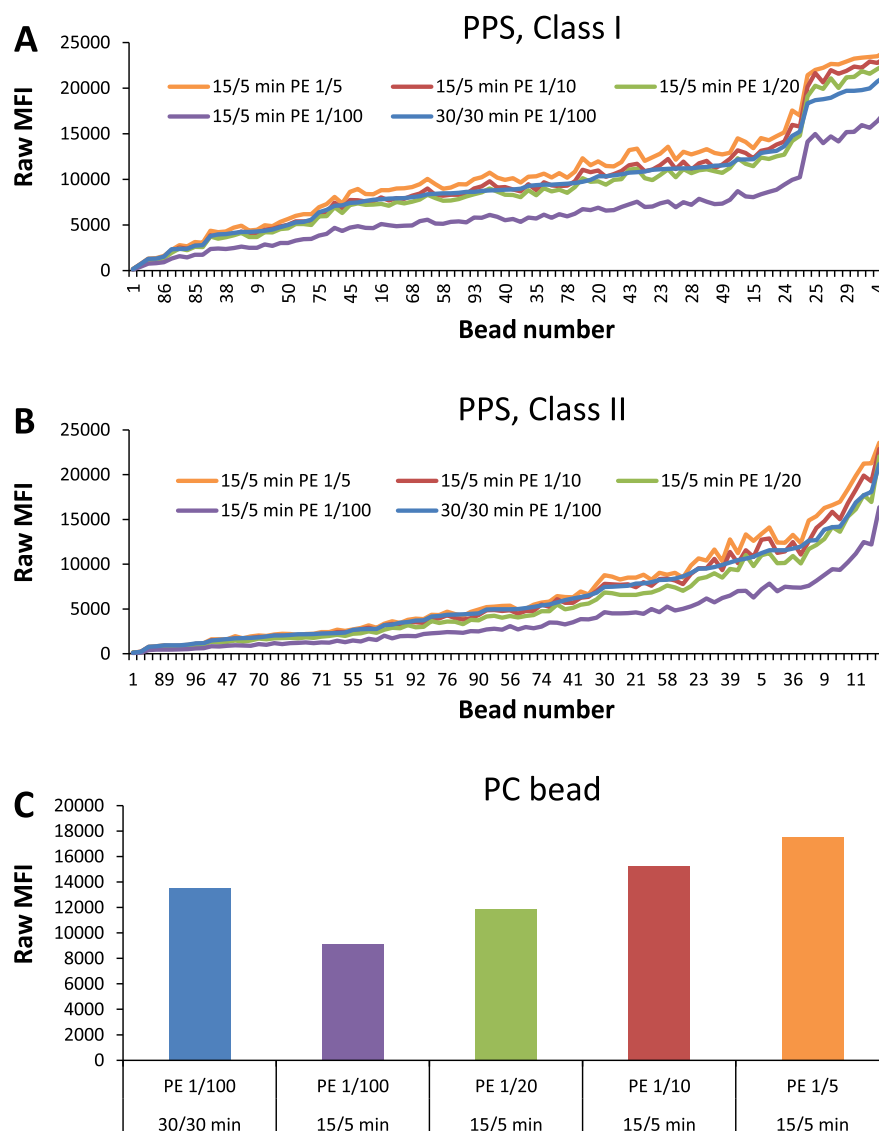


Fig. 4. Increased anti-IgG-PE concentration compensates for low MFI with the shortened incubation SAB protocol. PPS (A, B) sera were tested by HLA class I (A) or class II (B) SAB using the OLSAB protocol (30/30 min blue) vs short incubation protocol (15/5 min) with different concentrations of anti-IgG-PE antibody ranging from 1:100 to 1:5. PC bead raw MFI values (C) obtained with the OLSAB protocol vs short incubation protocol (15/5 min) tested with different concentrations of anti-IgG-PE (1:100 – 1:5) are shown. Representative raw MFI values from 1 of 3 independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were also obtained for sera analyzed at the SWID laboratory. Of the 42 SAB assay panels performed (22 HLA class I and 20 HLA class II), 28 were HLA antibody positive (16 class I and 12 class II) and 14 were antibody negative (6 class I and 8 class II). Again, the reactivity patterns observed for samples tested using the OLSAB procedure were consistent with those obtained using the ROB protocol, confirming that use of the optimized ROB protocol does not negatively impact the quality of the SAB assay results.

Importantly, linear regression analysis of baseline MFI values produced at the Halifax lab showed an excellent correlation between the protocols for both HLA class I ($r^2 = 0.9908$; Fig. 5c) and class II ($r^2 = 0.9845$; Fig. 5d) beads. The overall baseline MFI values were slightly increased with the ROB assay when compared to the OLSAB protocol (mean MFI increase of 308 ± 856) but there were no significant differences in regard to HLA antibody assignment. These results were corroborated by validation testing performed at the SWID laboratory, where excellent correlation was

also observed between the OLSAB and ROB protocols for both HLA class I ($r^2 = 0.981$; Fig. 6a) and class II ($r^2 = 0.9747$; Fig. 6b) antibodies. Finally, excellent MFI correlation for class I ($r^2 = 0.9843$; Fig. 5e) and class II ($r^2 = 0.9931$; Fig. 5f) HLA antibodies was also seen when 5 μ l/reaction (rather than 2.5 μ l/reaction) of SABs were used with both the ROB and OLSAB protocols testing five ASHI PT sera (AC470–474) in the Halifax lab. Similar results were observed by the SWID laboratory testing the same ASHI PT sera (class I $r^2 = 0.9939$; class II $r^2 = 0.9879$).

Although significant differences were not observed between the ROB and OLSAB assay results, 44 reactions (1.1 reactions per panel) were identified that fell on opposite sides of the 2000 MFI cutoff (Fig. 7a). Similar results were seen when a 1000 MFI positive cutoff was used (data not shown). The mean MFI difference between the protocols for these reactions was 447 ± 572 , which is within the expected run to run variation for the single antigen bead assay [23]. In addition, these differences would not be considered clini-

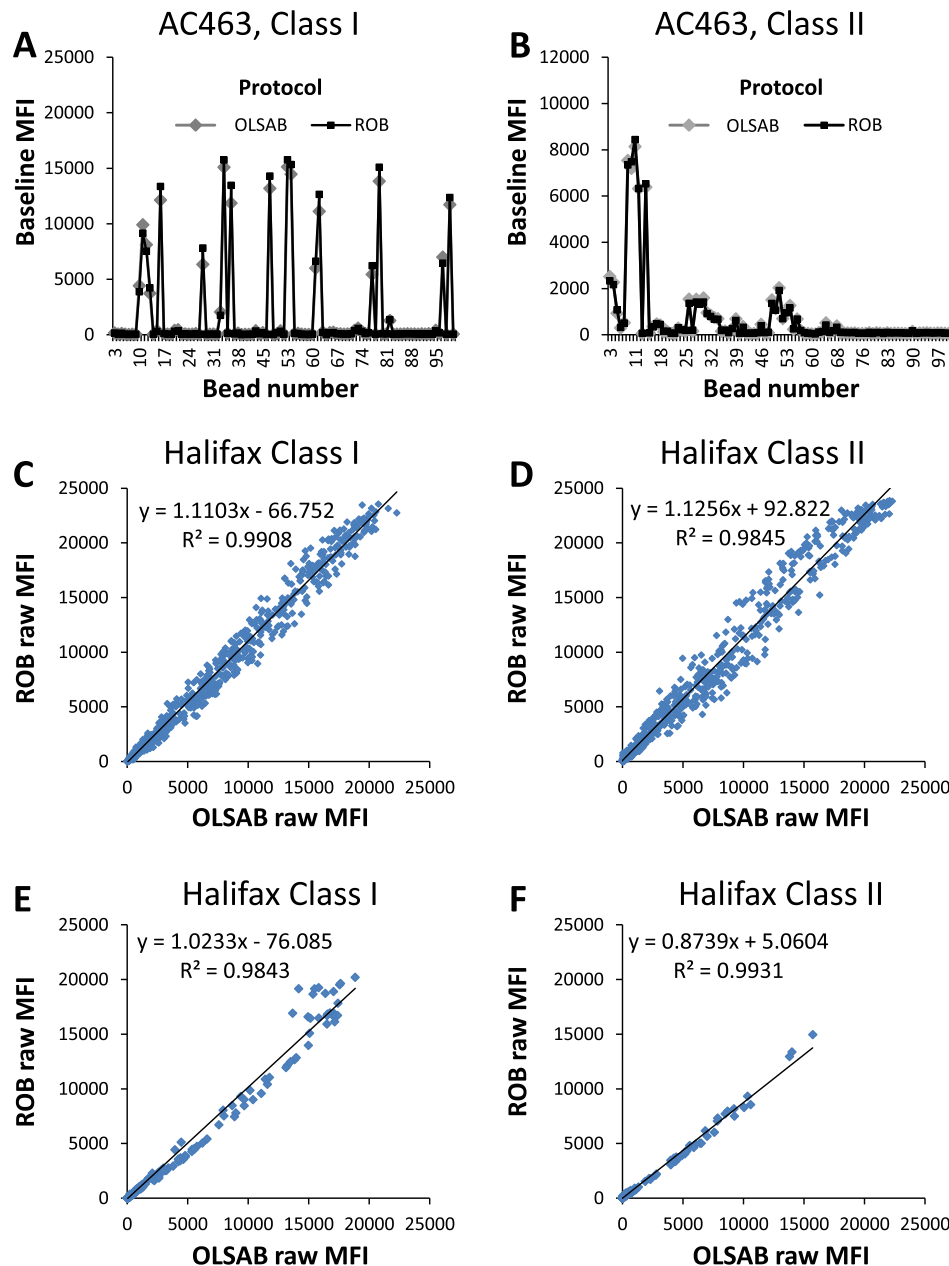


Fig. 5. There is excellent data correlation between the OLSAB assay and ROB protocol. Sera were tested for class I or class II antibody reactivity using the OLSAB or ROB protocols with either 2.5 μ l (A–D) or 5 μ l (E,F) SABs per reaction. (A,B) Show baseline MFI levels for HLA class I and class II beads treated with AC463 serum and tested with the OLSAB (grey) or ROB (black) protocol. (C,D) Show linear regression analysis comparing raw MFI values from the OLSAB and ROB protocols for 20 HLA class I and class II antibody positive panels from the Halifax laboratory. (E,F) Show linear regression analysis comparing raw MFI values from the OLSAB and ROB protocols for AC470–474 class I and class II SAB assay results.

cally significant as multiple factors in addition to MFI levels, including epitope analysis and comparison to antibodies identified in historical sera, must be taken into account when assigning positive antibody specificities. An example of an antibody reaction spanning the 1000 MFI cutoff is shown in Fig. 7b, in which both the OLSAB and ROB protocols show a reactivity pattern consistent with the presence of a weak Aw4/Bw4 epitope-specific antibody (highlighted with blue circles). Despite slight differences in MFI levels that surround the 1000 MFI cutoff, the antibody reactivity patterns reported for either assay would be identical as all antigens expressing the Aw4/Bw4 epitope would be considered positive based on epitope analysis.

3.5. Comparison of inter-laboratory results

To investigate the inter laboratory reproducibility of the ROB and OLSAB protocols, five proficiency testing samples (AC-460–464) were run in the Halifax and SWID HLA laboratories using both methods. Interestingly, there was a trend towards improved MFI correlation between the laboratories when the ROB protocol was used ($r^2 = 0.9816$ vs 0.9451 for the ROB and OLSAB protocols, respectively; Fig. 8a and b). The trend towards improved correlation was most evident for antibodies with MFI between 500 and 2000 ($r^2 = 0.7824$ vs 0.6313 for the ROB and OLSAB protocols, respectively; Fig. 8c and d), although results for antibodies with

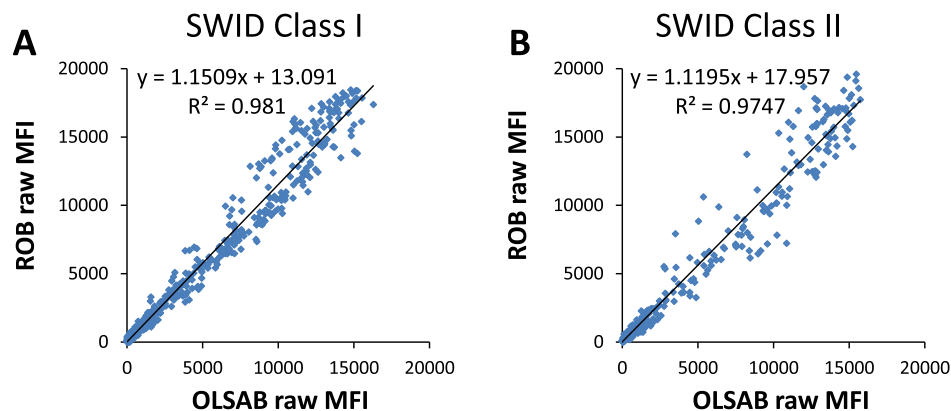


Fig. 6. A strong correlation between the OLSAB assay and ROB protocol is also seen at the SWID laboratory. Sera were tested for class I or class II antibody reactivity using the OLSAB or ROB protocols. (A,B) Show linear regression analysis comparing raw MFI values from the OLSAB and ROB protocols for 12 HLA class I and 9 class II antibody positive panels from the SWID laboratory.

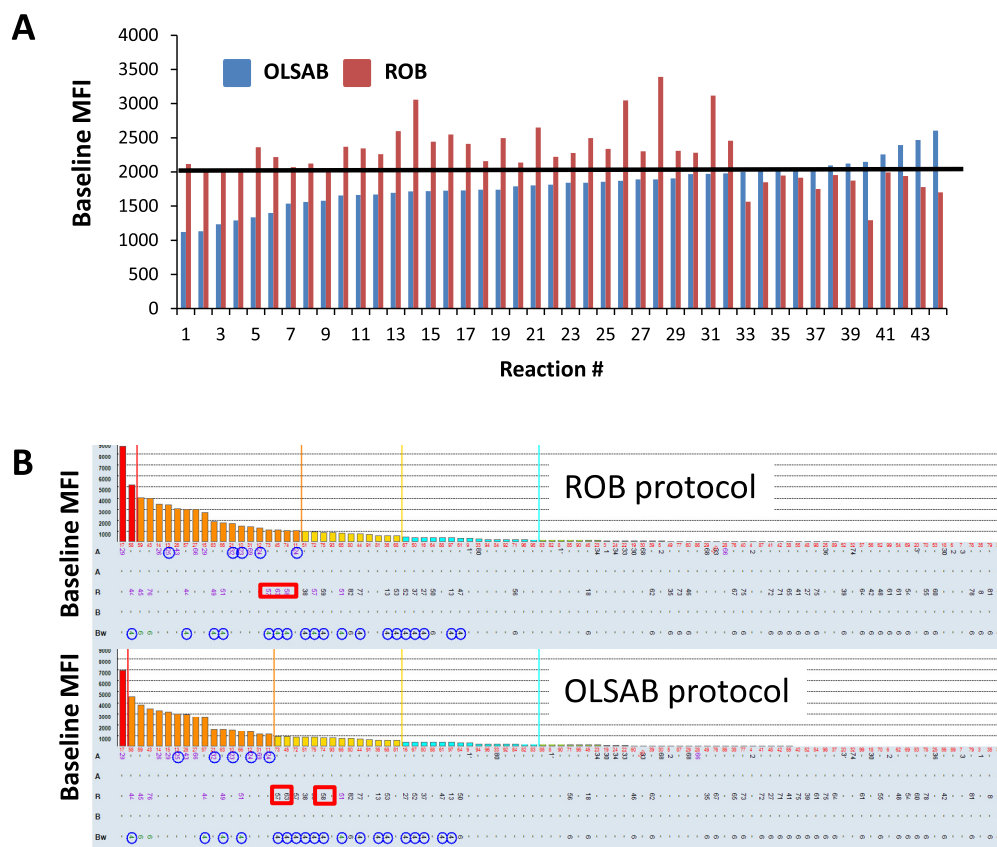


Fig. 7. Identification of OLSAB and ROB protocol reactions that span the MFI cutoff does not alter antibody assignment. (A) Reactions for the OLSAB (blue) and ROB (red) protocols that span the 2000 MFI cutoff (black line) are shown. (B) An example of differing reactivity around the 1000 MFI cutoff seen with the OLSAB and ROB protocol. HLA class I baseline MFI histograms of patient sera tested at the Halifax laboratory using the ROB (upper panel) or the OLSAB (lower panel) protocol, Aw4/Bw4 antigens are highlighted with blue circles. The three discordant bead reactions (B57, B58 and B63) around the 1000 MFI cutoff (orange line) are highlighted with red squares. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher MFI values (2,000–20,000) were also impacted ($r^2 = 0.9594$ vs 0.882 for the ROB and OLSAB protocols, respectively; Fig. 8e and f).

4. Discussion

The goal of this study was to develop a rapid LABScreen SAB assay protocol to expedite HLA antibody testing without compro-

mising the overall quality of the results and the signal to noise differential afforded by the LABScreen SAB method. This was accomplished by modifying three key assay parameters: (1) reducing centrifugation time during wash cycles (saving 20 min), (2) reducing serum and anti-human IgG-PE secondary antibody incubation times (saving 40 min) and (3) increasing the concentration of anti-human IgG-PE antibody to compensate for the decreased MFI seen with shortened incubations. The resulting rapid optimized SAB (ROB) protocol can be performed in less than 30 min,

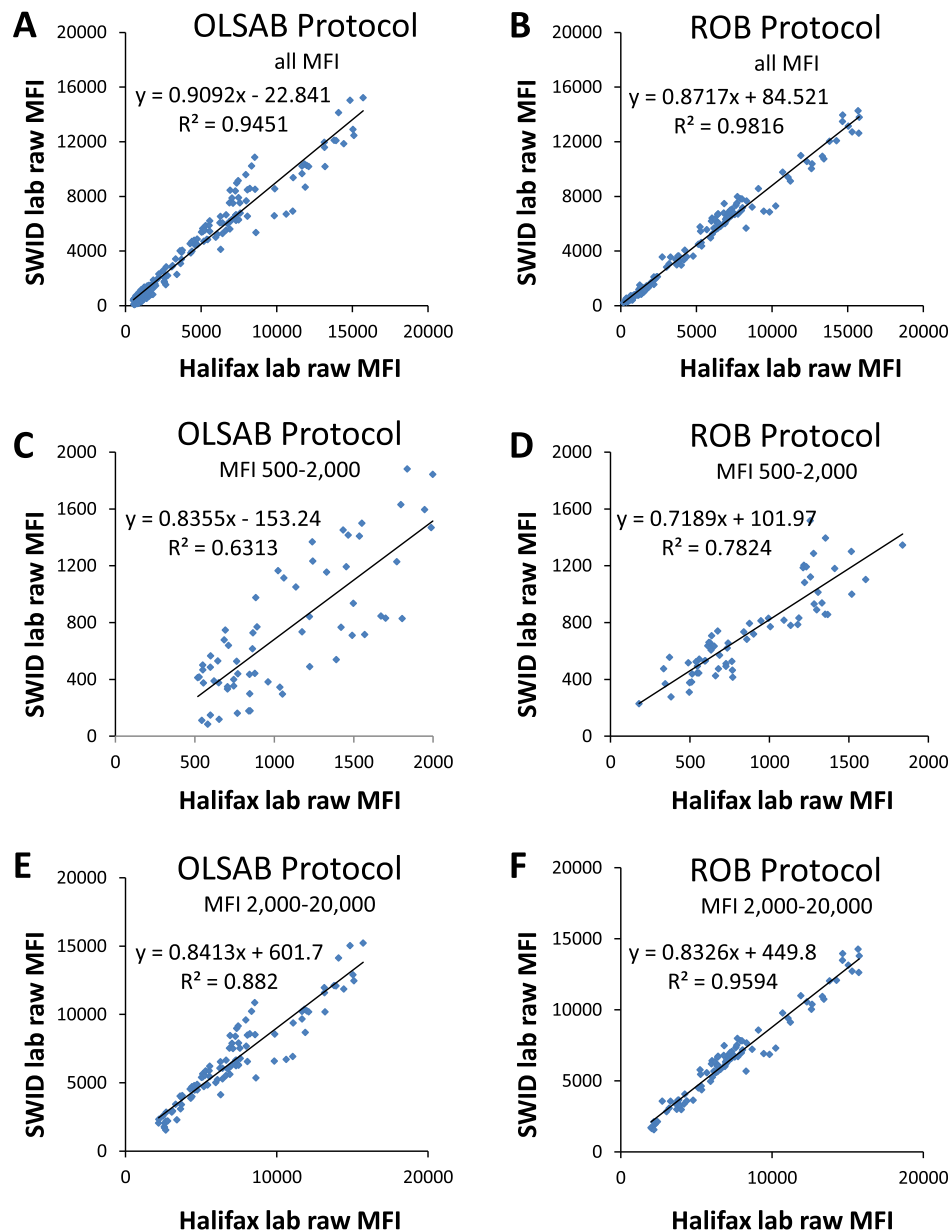


Fig. 8. Comparison of MFI data from ASHI samples AC-460–464 between the SWID laboratory and Halifax laboratory show excellent correlation. Linear regression analysis of raw MFI values from ASHI samples AC-460–464 tested at the SWID and Halifax laboratories by the OLSAB (A,C,E) and ROB (B,D,F) protocols. Linear regression analysis for all antibody MFI levels (A,B), antibodies with a raw MFI between 500 and 2000 (C,D), and antibodies with a raw MFI between 2000 and 20,000 (E,F) are shown.

representing a >70% reduction in assay time. Parallel testing of 58 sera at two different HLA laboratories showed that the ROB protocol generates results strongly concordant to those generated with the OLSAB method with an r^2 value of over 0.98, independent of the bead volume used (2.5 or 5 μ l). Only minor MFI differences were noted, which could be attributed to normal run to run variation [23]. Importantly, there were no significant differences in antibody specificity assignment.

The specific serum and secondary antibody incubation times (15 min and 5 min, respectively) selected for the SAB assay optimization in this study were based on preliminary time course experiments and our previous flow cytometry crossmatch protocol optimization data [24] with the overall goal to reduce assay time, while maintaining sensitivity. However, alternate incubation times could be selected based on specific laboratory preferences and/or needs. For example, our preliminary studies suggest that a protocol using 10 min incubations with both sera and secondary antibody

yields similar MFI results to the OLSAB and ROB methods. In addition, when optimal concentration of the secondary antibody is used (1:10), SAB MFI values do not vary significantly when single modifications are made to either serum incubation time (15 or 20 min instead of 30 min) or secondary antibody incubation time (5, 10, 15 or 20 min instead of 30 min). Therefore, several combinations of alternate incubation times could be used to obtain MFI results similar to the OLSAB or ROB protocols, provided that the secondary antibody is titrated appropriately.

Interestingly, the inter-laboratory comparisons of five ASHI PT sera showed a trend towards improved MFI concordance when SAB testing was performed using the ROB protocol than the OLSAB procedure. This trend was most evident for specificities in the 500–2000 MFI range, which spans the positive cutoff used by most HLA laboratories in North America. We have seen similar improvement in MFI concordance in a recent multicenter ROB protocol evaluation study that included ten centers in the US and Canada [28]. It

is unclear why the ROB protocol appears to improve inter-laboratory concordance of the results. It is possible that increase in anti-IgG-PE antibody concentration in the ROB protocol allows for a more uniform level of antibody binding when compared to the more dilute (1:100) antibody concentration used in the OLSAB assay.

Based on the validation results presented here, the ROB protocol was implemented in both the Halifax and SWID HLA laboratories and has been used in routine clinical testing for nearly 3 years. The usefulness of the ROB protocol was immediately noted in cases where antibody identification had to be performed on call during a deceased donor work-up or for late-in-the-day STAT requests. The decreased assay time afforded by the ROB protocol was also appreciated by laboratory technologists who found that the shortened procedure gave them more flexibility with regards to assay start time and provided them with additional time for data analysis and the reporting of test results. Importantly, neither laboratory has reported any significant changes in antibody reactivity patterns, MFI values, background reactivity, incidence of false positive or false negative reactions, antibody assignment, or performance in external proficiency testing. Furthermore, recently the ROB protocol was successfully revalidated by the Halifax laboratory using the current lots of LABScreen HLA class I (LS1A04 lot 10) and class II (LS2A01 lot 11) SABs and the LABScan 3D Luminex system, with no protocol adjustments required to achieve excellent MFI concordance with the OLSAB assay.

It was not surprising that 1 min centrifugation cycles were found to be sufficient to pellet the antigen-coated beads. Our previous work optimizing the flow cytometry crossmatch assay demonstrated that 1 min spins were sufficient to pellet lymphocytes [24]. In fact, we have been using 1 min washes with LABScreen SAB, LABType SSO and flow crossmatch assays for more than three years, with no impact on bead/cell counts or quality of results. Reducing centrifugation time is a simple and effective way of improving assay turn around time.

In our hands, the optimal anti-human IgG-PE antibody concentration for use with the ROB protocol was a 1:10 stock dilution as it showed the best MFI correlation with the OLSAB method. However, the optimal antibody dilution could be influenced by many factors such as the lot of secondary antibody or the lot of SABs used. Therefore, laboratories interested in validating this protocol should first perform a secondary antibody titration study to ensure the best possible MFI correlation with their existing method.

It is important to emphasize that all sera in our study were treated with EDTA before SAB testing. By chelating calcium, EDTA inhibits complement fixation and prevents any complement-mediated interference with the detection of high titer complement fixing HLA antibodies [20]. Complement-dependent interference with antibody detection may be sensitive to incubation time, therefore any sera that exhibit such interference may show a different pattern of reactivity when tested with the OLSAB (30 min serum incubation) vs the ROB (15 min serum incubation) protocol. This should be taken into consideration when validating the ROB protocol.

In summary, this paper describes the ROB protocol, an optimized version of the One Lambda LABScreen SAB assay that, not only decreases protocol time by over 70% without compromising assay sensitivity or accuracy, but also may improve inter-laboratory concordance of MFI. The OLSAB assay protocol appears to be based primarily on the standard flow cytometry crossmatch method [25], and a review of data presented in the original studies describing the LABScreen SAB assay suggest that assay conditions and reagent concentrations were not specifically optimized for this procedure [3,29–31]. Our work underscores the importance and usefulness of assay optimization to improve the quality of testing and decrease turn around time. This should be an important part of any test development and we urge product developers and man-

ufacturers to work closely with the HLA community to improve their products.

Author contributions

RL was responsible for the study concept, study design, data acquisition, analysis and interpretation. He also drafted and revised the manuscript. AG participated in data analysis, data interpretation, manuscript drafting, and revision. CM participated in data acquisition, data analysis, and manuscript revision. RB and HM participated in study design, data analysis and manuscript revision.

Conflicts of interest

The authors declare they have no conflicts of interest.

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References

- [1] P. Amico, G. Hönger, M. Mayr, J. Steiger, H. Hopfer, S. Schaub, Clinical relevance of pretransplant donor-specific HLA antibodies detected by single-antigen flow-beads, *Transplantation* 11 (2009) 1681–1688.
- [2] C. Lefaucheur, C. Suberbielle-Boissel, G.S. Hill, D. Nochy, J. Andrade, C. Antoine, C. Gautreau, D. Charron, D. Grotz, Clinical relevance of preformed HLA donor-specific antibodies in kidney transplantation, *Am. J. Transplant.* 2 (2008) 324–331.
- [3] LABScreen® Single Antigen HLA Class I and Class II, Product Insert, One Lambda Inc. <http://www.onelambda.com/>, 2017.
- [4] J.M. Cecka, Current methodologies for detecting sensitization to HLA antigens, *Curr. Opin. Organ Transplant.* 16 (2011) 398–403.
- [5] H.M. Gebel, R.A. Bray, The evolution and clinical impact of human leukocyte antigen technology, *Curr. Opin. Nephrol. Hypertens.* 19 (2010) 598–602.
- [6] H.M. Gebel, R.S. Liwski, R.A. Bray, Technical aspects of HLA antibody testing, *Curr. Opin. Organ Transplant.* 18 (2013) 455–462.
- [7] R.A. Bray, J.D. Nolen, C. Larsen, T. Pearson, K.A. Newell, K. Kokko, A. Guasch, P. Tso, J.B. Mendel, H.M. Gebel, Transplanting the highly sensitized patient: the emory algorithm, *Am. J. Transplant.* 6 (2006) 2307–2315.
- [8] C.J. Taylor, V. Kosmoliaptis, L.D. Sharples, D. Prezzi, C.H. Morgan, T. Key, A.N. Chaudhry, I. Amin, M.R. Clatworthy, A.J. Butler, C.J. Watson, J.A. Bradley, Ten-year experience of selective omission of the pretransplant crossmatch test in deceased donor kidney transplantation, *Transplantation* 89 (2010) 185–193.
- [9] A.W. Bingaman, C.L. Murphey, J. Palma-Vargas, F. Wright, A virtual crossmatch protocol significantly increases access of highly sensitized patients to deceased donor kidney transplantation, *Transplantation* 86 (2008) 1864–1868.
- [10] J.M. Cecka, A.Y. Kucheryavaya, N.L. Reinsmoen, M.S. Leffell, Calculated PRA: initial results show benefits for sensitized patients and a reduction in positive crossmatches, *Am. J. Transplant.* 11 (2011) 719–724.
- [11] L.A. Baxter-Lowe, M. Cecka, M. Kamoun, J. Sinacore, M.L. Melcher, Center-defined unacceptable HLA antigens facilitate transplants for sensitized patients in a multi-center kidney exchange program, *Am. J. Transplant.* 14 (2014) 1592–1598.
- [12] J.M. Blumberg, H.A. Gritsch, E.F. Reed, J.M. Cecka, G.S. Lipshutz, G.M. Danovitch, S. McGuire, D.W. Gjertson, J.L. Veale, Kidney paired donation in the presence of donor-specific antibodies, *Kidney Int.* 84 (2013) 1009–1016.
- [13] H.C. Sullivan, R.S. Liwski, R.A. Bray, H.M. Gebel, The road to HLA antibody evaluation: do not rely on MFI, *Am. J. Transplant.* (2017), <http://dx.doi.org/10.1111/ajt.14229>.
- [14] R.S. Liwski, H.M. Gebel, Of cells and microparticles: assets and liabilities of HLA antibody detection, *Transplantation* (2017) (in press), <http://dx.doi.org/10.1097/TP.0000000000001818>.
- [15] N. El-Awar, P.I. Terasaki, A. Nguyen, N. Sasaki, L.E. Morales-Buenrostro, H. Saji, E. Maruya, F. Poli, Epitopes of human leukocyte antigen class I antibodies found in sera of normal healthy males and cord blood, *Hum. Immunol.* 70 (2009) 844–853.
- [16] J. Visentin, G. Guidicelli, T. Bachelet, C. Jacquelinet, B. Audry, T. Nong, V. Dubois, J.F. Moreau, J.H. Lee, L. Couzi, P. Merville, J.L. Taupin, Denatured class I human leukocyte antigen antibodies in sensitized kidney recipients: prevalence, relevance, and impact on organ allocation, *Transplantation* 98 (2014) 738–744.
- [17] H.G. Otten, M.C. Verhaar, H.P. Borst, M. van Eck, W.G. van Ginkel, R.J. Hené, A.D. van Zuijlen, The significance of pretransplant donor-specific antibodies reactive

- with intact or denatured human leukocyte antigen in kidney transplantation, *Clin. Exp. Immunol.* 173 (2013) 536–543.
- [18] A.A. Zachary, D.P. Lucas, B. Detrick, M.S. Leffell, Naturally occurring interference in Luminex assays for HLA-specific antibodies: characteristics and resolution, *Hum. Immunol.* 70 (2009) 496–501.
- [19] V. Kosmoliaptsis, C. O'Rourke, J.A. Bradley, C.J. Taylor, Improved Luminex-based human leukocyte antigen-specific antibody screening using dithiothreitol-treated sera, *Hum. Immunol.* 71 (2010) 45–49.
- [20] M. Schnaidt, C. Weinstock, M. Jurisic, B. Schmid-Horch, A. Ender, D. Wernet, HLA antibody specification using single-antigen beads—a technical solution for the prozone effect, *Transplantation* 92 (2011) 510–515.
- [21] J. Visentin, M. Vigata, S. Daburon, C. Contin-Bordes, V. Fremeaux-Bacchi, C. Dromer, M.A. Billes, M. Neau-Cransac, G. Guidicelli, J.L. Taupin, Deciphering complement interference in anti-human leukocyte antigen antibody detection with flow beads assays, *Transplantation* 98 (2014) 625–631.
- [22] E. Schwaiger, M. Wahrmann, G. Bond, F. Eskandary, G.A. Böhmig, Complement component C3 activation: the leading cause of the prozone phenomenon affecting HLA antibody detection on single-antigen beads, *Transplantation* 97 (2014) 1279–1285.
- [23] E.F. Reed, P. Rao, Z. Zhang, H. Gebel, R.A. Bray, I. Guleria, J. Lunz, T. Mohanakumar, P. Nickerson, A.R. Tambur, A. Zeevi, P.S. Heeger, D. Gjertson, Comprehensive assessment and standardization of solid phase multiplex-bead arrays for the detection of antibodies to HLA, *Am. J. Transplant.* 13 (2013) 1859–1870.
- [24] R. Liwski, G. Adams, G. Peladeau, D. Eckels, R. Bray, H. Gebel, An optimized flow cytometric crossmatch assay expedites pre-transplant immunologic risk assessment, *Hum. Immunol.* 72 (2011) 48.
- [25] R.A. Bray, L.K. Lebeck, H.M. Gebel, The flow cytometric crossmatch. Dual-color analysis of T cell and B cell reactivities, *Transplantation* 48 (1989) 834–840.
- [26] R. Liwski, D. Pochinco, K. Tinkam, H. Gebel, P. Campbell, P. Nickerson, Canada-wide evaluation of rapid optimized flow crossmatch (ROFCXM) protocol, *Hum. Immunol.* 73 (2012) 26.
- [27] R. Liwski, D. Pochinco, K. Tinkam, P. Campbell, P. Nickerson, Going with the flow, Canadian crossmatch standardization, *Hum. Immunol.* 73 (2012) 28.
- [28] R. Liwski, P. Campbell, A. Colovai, D. Crowe, A. Halpin, L. Hidalgo, R. Kerman, P. Jindra, D. Li, J. Lunz, C. Murphey, P. Nickerson, D. Pochinco, S. Rosen-Bronson, O. Timofeeva, P. Warner, A. Zeevi, The test of time. A multicenter evaluation of the rapid optimized single antigen bead (ROB) protocol for LABScreen, *Hum. Immunol.* 76 (2015) 220.
- [29] R. Pei, G. Wang, C. Tarsitani, S. Rojo, T. Chen, S. Takemura, A. Liu, J. Lee, Simultaneous HLA class I and class II antibodies screening with flow cytometry, *Hum. Immunol.* 59 (1998) 313–322.
- [30] R. Pei, J. Lee, T. Chen, S. Rojo, P.I. Terasaki, Flow cytometric detection of HLA antibodies using a spectrum of microbeads, *Hum. Immunol.* 60 (1999) 1293–1302.
- [31] R. Pei, J.H. Lee, N.J. Shih, M. Chen, P.I. Terasaki, Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities, *Transplantation* 75 (2003) 43–49.