HISTO SPOT® AB Kits

Technical Report

Performance of the HISTO SPOT® HLA AB test in the Eurotransplant EPT and in the German INSTAND quality exchange test

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Introduction

The introduction of solid-phase immunoassays using recombinant single antigens (SA) for the detection and characterization of human leukocyte antigen (HLA) antibodies in transplantation has resulted in a new paradigm with respect to the interpretation of donor-specific antibodies (DSA). This due to the fact that the SA tests provide a much higher sensitivity than the complement dependent lymphocytotoxicity (CDC) assays. Although the SA assays have permitted the detection of antibodies not detectable by CDC, the clinical significance of these antibodies is incompletely understood (Roelen et al., 2012). Nevertheless, the detection of these antibodies has led to changes in the clinical management of sensitized patients. In addition, SA testing raises technical issues that require resolution and careful consideration when interpreting antibody results (Tait et al., 2013).

One of the problems reported with SA tests where the recombinant single antigens are bound to beads for the Luminex instrument is the detection of antibodies directed to denatured HLA antibodies which lack clinical relevance (Pereira et al., 2011; El-Awar et al. 2009, Poli et al. 2011, Jacob et al. 2011, Carrie et al. 2016).

When the first SA assays were established on the Luminex instrument there was no gold standard to compare the results to and still there is no method that is reproducible enough and sufficiently validated to clearly decide which reactions are truly positive. This is illustrated as well by the huge variability in reported HLA antibodies in the different quality assessment schemes (e.g. INSTAND, EPT Eurotransplant), where a consensus of between 85% and 95% of the participating labs defines a correct positive reaction and 25% of discrepancies are accepted according to EFI standards.

For this study, the serum samples used in the Eurotransplant External Proficiency Testing (EPT) and in the German INSTAND quality testing scheme were tested with the HISTO SPOT® HLA AB test. The results are compared to the consensus results for the other SA tests established in the market (LABScreen® Single Antigen, One Lambda and LIFECODES LSA Kit, Immucor). Additionally, the results are compared to the consensus results reported in ELISA/SPA tests or complement dependent SA tests when available.

The HISTO SPOT® HLA AB test uses mostly recombinant single antigen proteins that are spotted on the bottom of a microtiter plate well to define HLA antibodies, whereas the other two tests use microsphere beads coated with recombinant single antigens. The micro

ELISA assay for the HISTO SPOT® HLA AB test runs fully automated on the MR.SPOT® processor.

Material and methods

Test principle of the HISTO SPOT® HLA AB test

The HLA antibody detection process in the HISTO SPOT® HLA AB test is based on the interaction between the antibodies present in the sample and the antigen immobilized on the microarray. The antibodies specifically bind to their target antigen and are then recognized by a horse radish peroxidase conjugated anti-IgG. The presence of the antigen/antibody/anti-IgG product is detected by a coloured spot formed by Tetramethylbenzidine.

The resulting antibody signals (coloured dots in the bottom of each test well) are photographed by the MR.SPOT® processor and the image is transferred into the HISTO MATCH interpretation software. The image analysis software determines the colour intensity and the background intensity of each spot in the array. Then a Mean Colour Intensity (MCI) is calculated by subtracting the background value from the spot intensity. Based on spot intensity and the variability of the background the software gives a cut off value for each test, but results have to be reviewed and edited by the user.

Serum samples and test method

48 EPT sera from the Eurotransplant Reference Laboratory in Leiden and 35 INSTAND sera from the years 2015-2018 were tested with the HISTO SPOT® HLA AB class I and class II test according to the instructions for use. If there were unspecific reactions the test was repeated with the SERA PURE reagent which is used instead of the sample dilution buffer and reduces background reactions.

Analysis of the results

The concordance of the results with the consensus results for the other SA tests and with the SPA/ELISA tests (when available) was determined. The results are given on the level of resolution defined by the published consensus results. For class I Cw18 and for class II DQ5 are excluded from the analysis because the respective antigens are not present on the HISTO SPOT® chips used at the time.

If there are two antigens on the chip for one serological specificity (e.g. A*02:01 and A*02:03 for A2) and only one of the antigens is positive, the serological specificity was classified as positive.

The following parameters are calculated:

- Percentage of concordant consensus specificities (= % Concordance)

The number of consensus specificities (defined by EPT/INSTAND) with a given method that are detected with the HISTO SPOT® HLA AB test, too, is determined and related to the total number of consensus specificities. This gives a measure of the sensitivity of the HISTO SPOT® HLA AB test in comparison to other methods. This is done for complete sets of sera, for individual sera and for single serological specificities to find out if sources for discordance can be identified.

- Number of additional specificities detected (= Additional specificities)

Usually, the HISTO SPOT® HLA AB test identifies antibody reactions against

specificities that do not reach the consensus level, but are detected by other labs as well and, therefore, most likely not false positive. Positive reactions are classified into this category if they are reported by more than 5% of the participating labs for the EPT sera and by more than one lab for the INSTAND sera. This number might be an indicator for sera with a high level of weak antibodies or with unspecific background reactivity.

- Presumably false positive

Positive reactions are assumed to be most likely false positive if they were reported by less than 5% of the participating labs (EPT) or less than 2 labs (INSTAND). The percentage of presumably false positive reaction was calculated in relation to the number of non-consensus specificities that were negative with the HISTO SPOT® HLA AB test as well. This can be considered as a measure of the specificity of the test.

Results

EPT 2015-2018:

Two sera from EPT (EPT 2015-I and EPT 2017-A) for class I and one serum for class II (EPT 2018-B) were excluded because results were unspecific positive with all the antigens on the chip.

In total 1142 class I consensus specificities (reported by at least 95% of the labs) have been reported for the 46 sera that give interpretable results in this study. 82% of these specificities were detected by the HISTO SPOT® test as well. 139 additional specificities were detected that did not reach the consensus level, but were reported by more than 5% of the participating labs. 38 specificities were detected by the HISTO SPOT® HLA AB test but not with the other SA tests on the Luminex instrument (reported by less than 5% of the labs). For class II 88% of the 186 consensus specificities were detected by the HISTO SPOT® HLA AB test and 30 additional non consensus specificities. 20 additional specificities were found with the HISTO SPOT®HLA AB test, but not with the other SA antigen tests (Table 1).

Table 1: Specificities detected in the EPT sera 2015-2018

	Class I	**Class II**
Total no. of consensus specificities (95% of labs)	1142	186
% Concordance	82%	88%
Additional specificities	139	30
Presumably false positive	38	20

The level of concordance with the consensus was not the same for all the serological specificities that were analysed. A group of 19 serological specificities for class I and 6 specificities for class II showed a low concordance of less than 75%. These specificities are listed in Table 2 below.

The concordance with the consensus also varied considerably between sera (36%-100% for class I and 33%-100% for class II, see Figure 2 for class I).

Table 2: List of specificities (antigens) with a concordance below 75%

Class I	No. of pos. sera (consensus)	% concordance	% false positive
B75	19	68%	4%
B65	9	67%	0%
B71	15	67%	0%
Cw12	3	67%	10%
B46	14	64%	0%
B82	16	63%	0%
A11	10	60%	0%
A29	12	58%	0%
B8	13	54%	0%
A80	8	50%	3%
B27	22	50%	0%
B67	6	50%	0%
Cw17	8	50%	0%
B59	21	48%	0%
B39	9	33%	0%
Cw16	3	33%	10%
B73	14	29%	0%
Cw2	11	27%	3%
B37	25	24%	0%

Class II	No. of pos. sera (consensus)	% Concordance	% false positive
DQ2	2	67%	2%
DR15	6	67%	0%
DR7	3	60%	0%
DR16	3	50%	0%
DR53	2	50%	5%
DQ4	1	17%	3%

Low concordance together with high level of false positive reactions: possibly low specificity of the antigen on the chip

High number of positive sera together with very low concordance and few false positives: lack of reactivity of the antigen on the chip or over-reactivity of the antigens on the Luminex beads

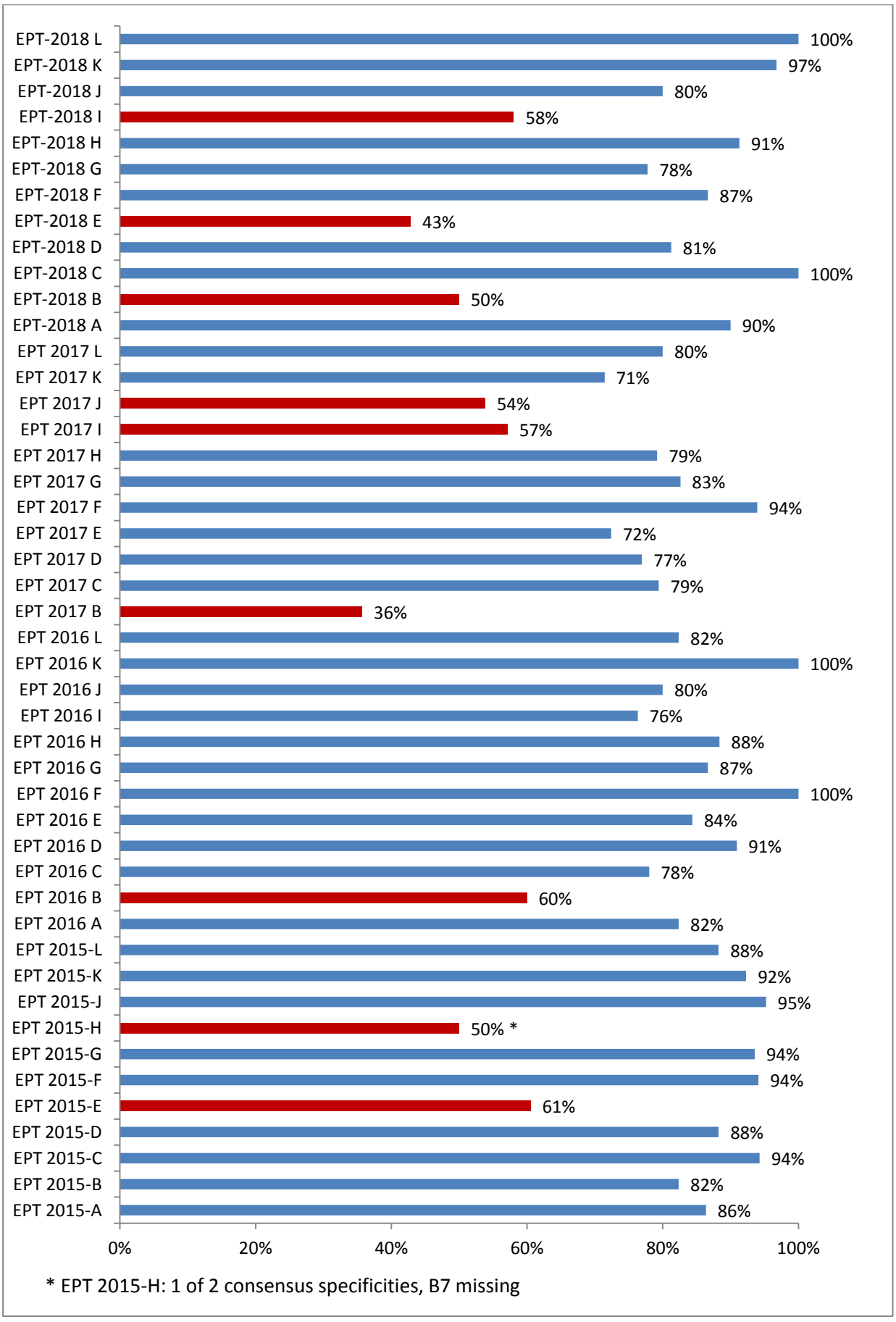

Figure 2: Concordance of the results for the HISTO SPOT® HLA AB test with the 95% consensus results for EPT sera

INSTAND 2015-2018:

One serum for class II (INSTAND Jan 15 - 25) was excluded from the analysis because results were unspecific positive with all the antigens on the chip.

The two SA tests for the Luminex instrument (LABScreen® Single Antigen, One Lambda = OLI and LIFECODES LSA Kit, Immucor = Immu) were evaluated in two different categories by INSTAND in 2015 and 2016. From 2017 both tests were combined in one category (Lumi). Therefore, the data set for the INSTAND sera was divided into two periods. The number of consensus specificities reported with the LIFECODES LSA Kit was considerably lower than with the LABScreen® SA test for class I, i.e. the LIFECODES LSA Kit found 84% of the consensus specificities reported with the LABScreen® SA test. For class II both tests detected the same number of consensus specificities. The PRA tests detected less than half of the specificities reported with the SA tests for both, class I and class II (Figure 3). The concordance of the HISTO SPOT® HLA AB test with the consensus results is shown in Figure 4. For class I, the HISTO SPOT® HLA AB test detected 67% of the consensus specificities reported for the LABScreen® Single Antigen test and 77% of the ones reported for LIFECODES LSA kit. The HISTO SPOT® HLA AB test seems to be closer to the PRA tests in respect to sensitivity with 91% concordance, but there are 10% "presumably false positive" reactions if the test is compared to the group of PRA tests. The same tendency is visible for the smaller set of sera from 2017 and 2018 when both SA tests were combined.

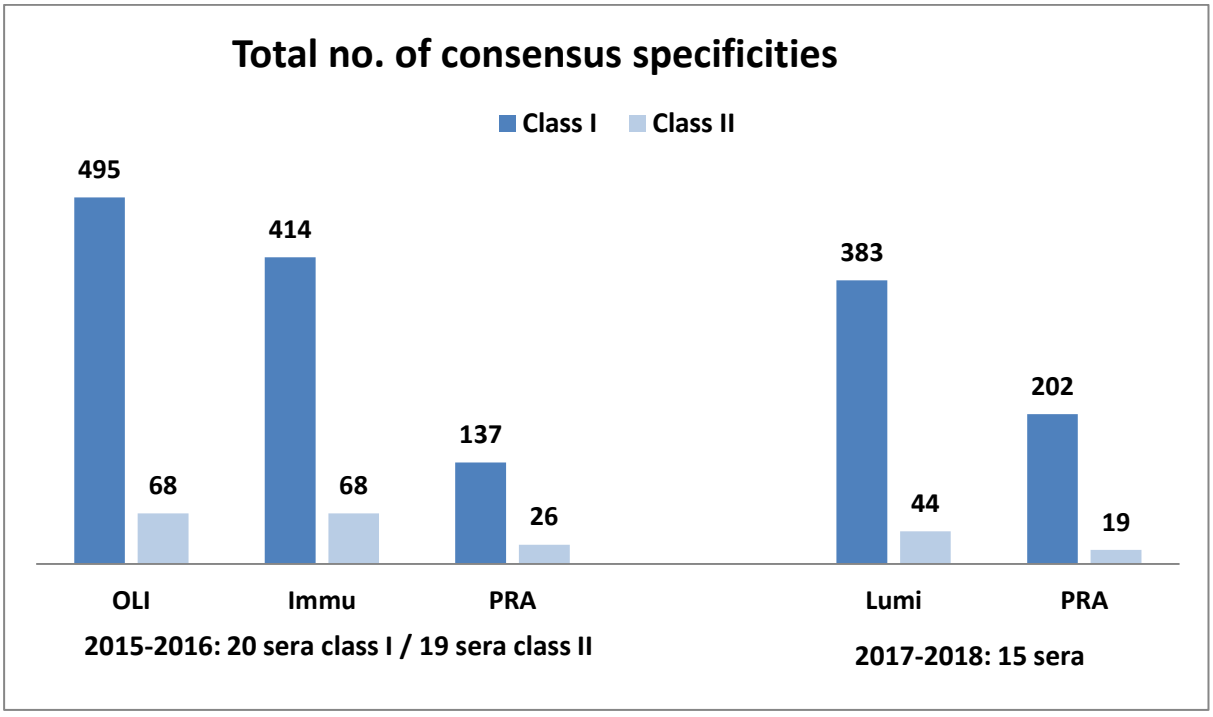

Figure 3: Total number of INSTAND consensus specificities
OLI = LABScreen® Single Antigen, One Lambda; Immu = LIFECODES LSA Kit, Immucor , Lumi = both SA tests combined, PRA = ELISA assays

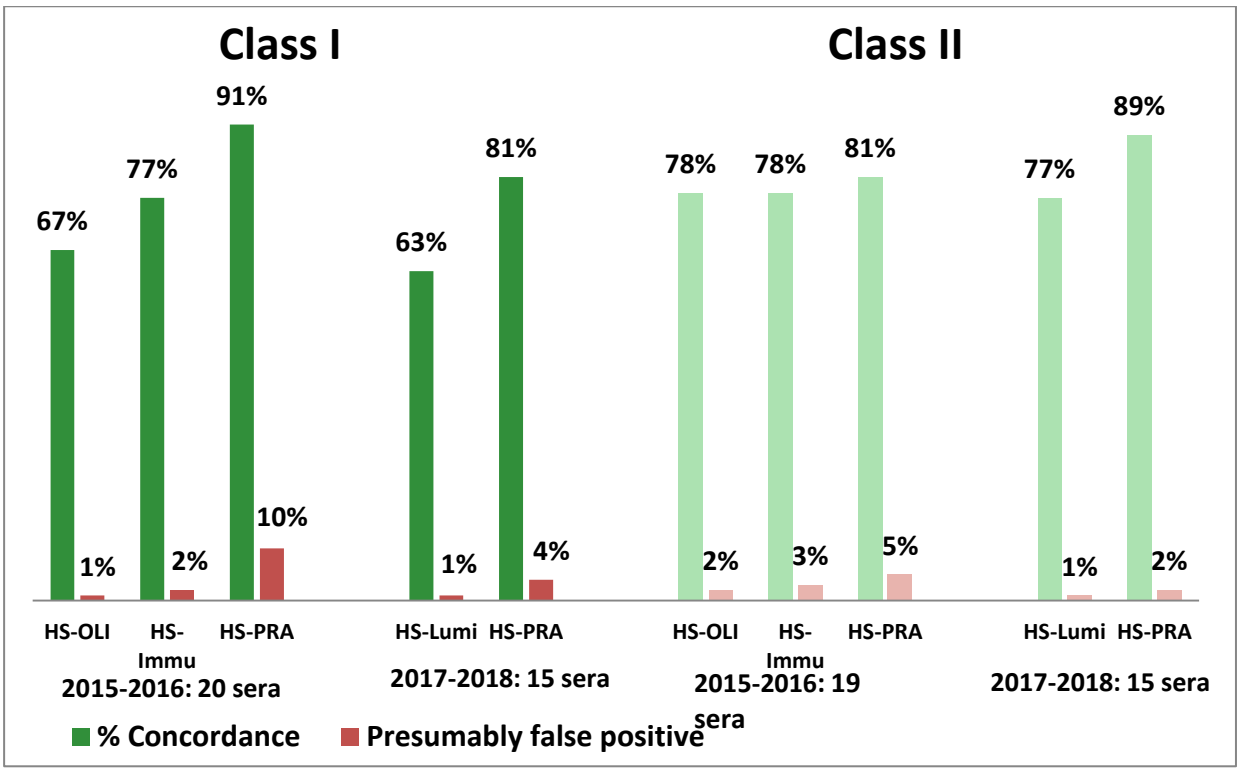

Figure 4: Concordance of the HISTO SPOT® HLA AB test with the INSTAND consensus results
HS = HISTO SPOT® HLA AB, OLI = LABScreen® Single Antigen, One Lambda; Immu = LIFECODES LSA Kit, Immucor, Lumi = both SA tests combined, PRA = ELISA assays

For Class II there is no difference between the two other SA tests and the concordance with the HISTO SPOT® HLA AB test is 78% and 77% for the two subsets of sera. Again, there is a higher concordance with the PRA tests for class II as well, but also a slightly higher number of additional specificities that are not detected by the PRA test but by the HISTO SPOT® HLA AB test. These are mostly detected by the two other SA tests as indicated by the very low number of "presumably false positive" reactions of the HISTO SPOT® HLA AB test found there.

Discussion

The results show that there is generally a high degree of variability in the results depending on the method and even between different vendors per method. From the results comparison alone it cannot be concluded if the "less sensitive" method misses HLA antibodies and has, therefore, false negative results or if the "more sensitive" method detects artefacts like "natural antibodies" (Morales-Buenrostro et al., 2008) or HLA antibodies against denatured HLA antigens (Ravindranath et al. 2017) which should rather be considered false positive.

Some of the specificities that showed a low concordance with the consensus results were mentioned in the literature about "natural antibodies". Antibodies against A80, B8, B27, B82 or Cw17 were found in healthy males without any immunizing events (Morales-Buenrostro et al., 2008, Gombos et al. 2013). Gombos et al. (2013) additionally found that in 77% of the patients without a history of immunizing events the Luminex SA test found antibodies whereas 98% of these patients were negative in ELISA and CDC. These "natural" antibodies are most likely directed against denatured antigens on

the beads which expose epitopes that are not accessible in the natural three dimensional structure of the HLA protein. Comparative tests with the LABScreen® Single Antigen test and the HISTO SPOT® HLA AB test show that the HISTO SPOT® HLA AB does not detect antibodies against denatured HLA antigens that are found with the LABScreen® Single Antigen test (Smith J, Poster presentation on BSHI 2018 and personal communication). As the antibodies against denatured antigens are not assumed to be clinically relevant the results with the HISTO SPOT® HLA AB test seem to be the more significant ones in this case.

Some of the differences between the SA tests might also be explainable because the allelic antigens for a serological specificity are not always the same in the different SA tests. This is not visible in the reported results that are on the serological level only.

Another source of variability that might explain part of the differences in concordance with the consensus found between sera are unspecific reactions of substances other than HLA antibodies that react with the surface on which the antigens are bound. Though the Luminex bead and the test wells in the HISTO SPOT® HLA AB test are both polystyrene the coating of the beads and the test wells will most likely be different.

Especially for antigens that show a low concordance with the consensus and a high number of “false positive” reactions (Cw12 and Cw16 for the EPT sera) an optimization of the recombinant proteins might improve results.

Interestingly, the difference between the two SA tests on the Luminex instrument is much smaller for class II than for class I and the concordance of the HISTO SPOT® HLA AB test with both tests 88% for the EPT sera and 78% with the INSTAND sera. The HISTO SPOT® HLA AB class II test used for this study still contains a few native proteins and a few suboptimal recombinant proteins that will be improved in the next lots.

Further studies on the clinical relevance and the differences found between the different SA tests are obviously needed to assess the advantages and disadvantages of the different techniques. The HISTO SPOT® HLA AB test should be treated as a category on its own in external proficiency testing until more data are available, because using the consensus derived from the SA tests on the Luminex instrument that are the most common one on the market will be misleading because the majority result is not necessarily the correct one.

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