HISTO SPOT® AB Kits – Technical Report

Reproducibility of the results with the HISTO SPOT® HLA AB test

Dr. Kristin Launhardt, BAG Health Care GmbH, Lich, Germany
Virginie Lefevre, innobiochips, Lille, France
Vianney Souplet, innobiochips, Lille, France

Introduction

This study has the aim to show reproducibility of results when testing sera for HLA antibodies with the HISTO SPOT® HLA AB test. The HISTO SPOT® HLA AB test is a microtiter plate-based test that runs on an automated bench top system, the MR.SPOT® processor. This assay can detect class I or class II antibodies via immobilized recombinant single antigens. The recombinant antigens are spotted on the bottom of the test wells – one test well contains all the antigens for either class I or class II. Bound antibodies are detected by a secondary antibody against whole human IgG and made visible by a color reaction. The result is documented by an image of the test well.

The reproducibility is an essential parameter for an accurate and stable test. The same serum should show the same pattern of positive reactions for HLA antibodies when tested at different times, by different users on different instruments and with different batches. The automation of the assay helps to improve standardization. Moreover, if a semiquantitative result is needed - for example to monitor the development of donor-specific antibodies after transplantation – the intensity of the signal should show a limited variability from batch to batch for a given serum.

Two approaches were chosen to determine the reproducibility of results. The first one was to let 11 different laboratories test the sera from the Eurotransplant external proficiency testing (EPT) 2019. For the determination of HLA antibodies based on single antigens the correct results were determined by 95% consensus results from 67 labs all using a single antigen beads (SAB) method on the Luminex instrument. Therefore, it basically measures the accordance of results obtained by a specific method in different labs. As there is no "gold standard" to define the true results and it is known that the Luminex method produces false positive (not clinically relevant) results due to denatured antibodies and detects "natural antibodies" in healthy males without an immunizing history, the Luminex consensus results should not be regarded as a reference that should be completely reproduced with a different single antigen method like the HISTO SPOT® HLA AB test.

For the purpose of this study, a 75% consensus result was determined because a 95% consensus would require that the results from all 11 labs match and this does not make sense. Then the number of discrepancies was analyzed for both methods as a measure for inter-lab reproducibility.

For the semiquantitative analysis the coefficient of variation of the signal intensity was analyzed for the same results from the EPT 2019 and were compared to QC results from different batches run on the same instrument in the same lab.

Materials and methods

The 12 sera from the EPT 2019 were sent out to 11 labs using the MR.SPOT® processor either for SSO typing or for HLA antibody analysis. As there were many untrained first-time users among these labs the qualitative interpretation was done at BAG Diagnostics according to the interpretation guidelines to avoid discrepancies caused by interpretation mistakes. The analysis included 4 different batches of the HISTO SPOT® HLA AB class I test covering 96 antigens.

Specificities found by at least 9 of the 11 labs were considered positive (75% consensus result).

Discrepancies were classified as specified in Table 1. These numbers were compared to the same parameters obtained from the 67 labs using the Luminex method, though this is only a rough comparison due to the higher number of labs and the 95% consensus used.

Table 1: Definition of results categories for the EPT 2019 sera

Result category	HISTO SPOT® HLA AB	Luminex method
Positive specificity	75% consensus	95% consensus
Questionable specificitiy	found by 2-8 labs	found by < 75% and >5% of the labs
False positive specificity	found by only 1 lab	found < 5% of the labs
False negative specificity	consensus specificity not found by a lab	consensus specificity not found by a lab

For the semiquantitative analysis the following parameter were analysed:

- **Mean:** mean pixel intensity of a spot measured by the image analysis module of the interpretation software
- **Background:** mean pixel intensity of the area around a spot
- **Signal**: the Mean value with the pixel intensity of the background around the spot subtracted.
- **S/B ration**: the Mean value divided by the pixel intensity of the background around the spot.

For each serum the coefficient of variation (CV) was calculated from the values of the 11 labs for each antigen. The global CV for each serum was calculated as the mean CV of the 96 antigens on the chip.

The same analysis was performed for 32 sera with 8 batches tested for quality control purposes. They were tested on the same MR.SPOT® instrument, but at different times in different runs

Results

Qualitative analysis of results for EPT sera:

The interpretation of the results gave 302 positive consensus specificities based on the reactivity of the 96 recombinant single antigens. 87 specificities came out as questionable. In total there were 68 false negative results and 26 false positive results which results in an average discrepancy rate of 2.83% per lab in relation to the number of consensus specificities. Figure 1 exemplary shows the detailed results for the serum EPT 2019 H. Questionable results are indicated there and false positive or false negative discrepancies are highlighted. In Figure 2 the array images for the same serum from labs 1 and 11 are shown to illustrate how questionable reactions and discrepancies looked like. Lab 1 had two false positive discrepancies which are very weak. It also illustrates that the questionable reactions are generally weak ones which are sometimes weakly visible and sometimes not.

Lab No	1	2	3	4	5	6	7	8	9	10	11	
Lot	112	112	121	122	113	112	112	112	113	113	112	Result
A*01:01	-	-	-	-	-	-	-	-	-	-	-	n
A*02:01	+	+	+	+	+	+	+	+	+	+	+	c
A*02:03	+	+	+	+	+	+	+	+	+	+	+	c
A*03:01	-	-	-	-	-	-	-	-	-	-	-	n
A*11:01	-	-	-	-	-	-	-	-	-	-	-	n
A*23:01	-	-	-	-	-	-	-	-	-	-	-	n
A*24:02	+	-	-	-	-	+	-	-	-	-	-	q
A*24:03	+	-	+	-	-	+	+	-	-	-	-	q
A*25:01	+	+	+	+	+	+	+	+	+	+	+	c
A*26:01	+	+	+	+	+	+	+	+	+	+	+	c
A*26:02	+	+	+	-	+	+	+	-	+	+	+	c
A*29:02	+	+	+	-	+	+	-	-	+	-	-	q
A*30:01	-	-	-	-	-	-	-	-	-	-	-	n
A*30:02	-	-	-	-	-	-	-	-	-	-	-	n
A*31:01	+	+	+	+	+	+	+	+	+	+	+	c
A*32:01	+	+	+	+	+	+	+	+	+	+	+	c
A*33:01	+	+	+	+	+	+	+	+	+	+	+	c
A*33:03	+	+	+	+	+	+	+	+	+	+	+	c
A*34:01	+	+	+	+	+	+	+	+	+	+	+	c
A*36:01	-	-	-	-	-	-	-	-	-	-	-	n
A*43:01	+	+	+	+	+	+	+	+	+	+	+	c
A*66:01	+	+	+	+	+	+	+	+	+	+	+	c
A*66:02	+	+	+	+	+	+	+	+	+	+	+	c
A*68:01	+	+	+	+	+	+	+	+	+	+	+	c
A*68:02	+	+	+	+	+	+	+	+	+	+	+	c
A*69:01	+	+	+	+	+	+	+	+	+	+	+	c
A*74:01	+	+	+	+	+	+	+	+	+	+	+	c
A*74:03	+	+	+	+	+	+	+	+	+	+	+	c
A*80:01	-	-	-	-	-	-	-	-	-	-	-	n
B*07:02	+	+	+	+	+	+	+	+	+	+	+	c
B*07:03	-	-	-	-	-	-	-	-	-	-	-	n
B*08:01	-	-	-	-	-	-	-	-	-	-	-	n
B*13:02	-	-	-	-	-	-	-	-	-	-	-	n
B*14:01	-	-	-	-	-	-	-	-	-	-	-	n
B*14:02	-	-	-	-	-	-	-	-	-	-	-	n
B*15:01	+	+	+	-	-	+	+	-	-	-	+	q
B*15:02	-	-	-	-	-	-	-	-	-	-	-	n
B*15:03	F+	-	-	-	-	-	-	-	-	-	-	n
B*15:09	-	-	-	-	-	-	-	-	-	-	-	n
B*15:12	-	-	-	-	-	-	-	-	-	-	-	n
B*15:13	-	-	-	-	-	-	-	-	-	-	-	n
B*15:16	+	+	+	+	+	+	+	+	+	+	+	c
B*15:17	+	+	+	+	+	+	+	+	+	+	+	c
B*15:18	-	-	-	-	-	-	-	-	-	-	-	n
B*18:01	-	-	-	-	-	-	-	-	-	-	-	n
B*27:05	+	-	-	-	-	-	+	-	-	-	-	q
B*27:08	+	+	+	+	+	+	+	+	+	+	+	c
B*35:01	-	-	-	-	-	-	-	-	-	-	-	n

Lab No	1	2	3	4	5	6	7	8	9	10	11	
Lot	112	112	121	122	113	112	112	112	113	113	112	Result
B*37:01	-	-	-	-	-	-	-	-	-	-	-	n
B*38:01	-	-	-	-	-	-	-	-	-	-	-	n
B*39:01	-	-	-	-	-	-	-	-	-	-	-	n
B*39:06	-	-	-	-	-	-	-	-	-	-	-	n
B*40:01	-	-	-	-	-	-	-	-	-	-	-	n
B*40:02	F+	-	-	-	-	-	-	-	-	-	-	n
B*41:02	-	-	-	-	-	-	-	-	-	-	-	n
B*42:01	+	+	+	+	+	+	+	+	+	+	+	c
B*44:02	-	-	-	-	-	-	-	-	-	-	-	n
B*44:03	-	-	-	-	-	-	-	-	-	-	-	n
B*45:01	-	-	-	-	-	-	-	-	-	-	-	n
B*46:01	+	+	+	+	+	+	+	+	+	+	+	c
B*47:01	-	-	-	-	-	-	-	-	-	-	-	n
B*48:01	-	-	-	-	-	-	-	-	-	-	-	n
B*49:01	+	+	+	-	-	+	+	-	-	-	-	q
B*50:01	+	-	-	-	-	+	-	-	-	-	-	q
B*51:01	-	-	-	-	-	-	-	-	-	-	-	n
B*51:02	-	-	-	-	-	-	-	-	-	-	-	n
B*52:01	+	+	+	-	-	+	-	-	-	-	-	q
B*53:01	-	-	-	-	-	-	-	-	-	-	-	n
B*54:01	+	+	+	+	+	+	+	+	+	+	+	c
B*55:01	+	+	+	+	+	+	+	+	+	+	+	c
B*56:01	+	+	+	+	+	+	+	+	+	+	+	c
B*57:01	+	+	+	+	+	+	+	+	+	+	+	c
B*58:01	+	+	+	+	+	+	+	+	+	+	+	c
B*59:01	-	-	-	-	-	-	-	-	-	-	-	n
B*67:01	+	+	+	+	+	+	+	+	+	+	+	c
B*73:01	+	+	+	F-	+	+	+	+	+	+	+	c
B*78:01	-	-	-	-	-	-	-	-	-	-	-	n
B*81:01	+	+	+	+	+	+	+	+	+	+	+	c
B*82:01	+	+	+	+	+	+	+	+	+	+	+	c
C*01:02	+	+	+	+	+	+	+	+	+	+	+	c
C*02:02	-	-	-	-	-	-	-	-	-	-	-	n
C*03:03	-	-	-	-	-	-	-	-	-	-	-	n
C*03:04	+	+	+	+	+	+	+	+	+	+	+	c
C*04:01	-	-	-	-	-	-	-	-	-	-	-	n
C*05:01	-	-	-	-	-	-	-	-	-	-	-	n
C*06:02	-	-	-	-	-	-	-	-	-	-	-	n
C*07:01	-	-	-	-	-	-	-	-	-	-	-	n
C*07:02	-	-	-	-	-	-	-	-	-	-	-	n
C*08:01	+	+	+	+	+	+	+	+	+	+	+	c
C*08:02	+	+	+	+	+	+	+	+	+	+	+	c
C*12:03	-	-	-	-	-	-	-	-	-	-	-	n
C*14:02	+	+	+	+	+	+	+	+	+	+	+	c
C*14:03	+	+	+	+	+	+	+	+	+	+	+	c
C*16:01	+	+	+	-	-	+	+	-	-	+	-	q
C*17:01	-	-	-	-	-	-	-	-	-	-	-	n
C*18:01	-	-	-	-	-	-	-	-	-	-	-	n

Figure 1: **Detailed qualitative results for serum EPT 2019 H from 11 labs**

n = negative, c = consensus specificity, q = questionable, + = positive reaction, - = negative reaction,
F- = false negative, F+ = false positive

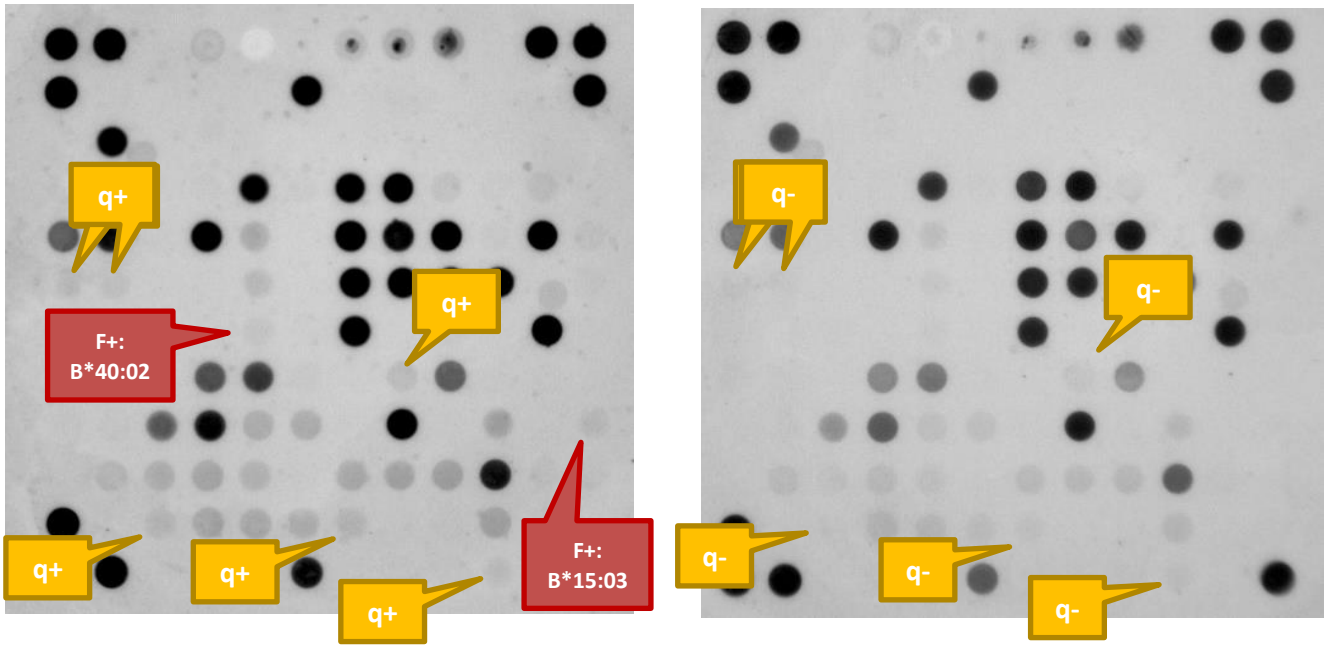


Figure 2: Array images for serum EPT 2019 H from lab 1 and lab 11

F- = false negative, F+ = false positive, q+ = questionable specificity positive, q+ = questionable specificity negative

To compare these results to the Luminex results reported to Eurotransplant the consensus specificities were defined based on serological specificities. This yields a lower number of consensus specificities because e.g. A*30:01 and A*30:02 were reported together as A30(19) if either one or both antigens were positive.

With the Luminex test more consensus specificities were found than with the HISTO SPOT® test, but at the same time the Luminex test gave more questionable reactions which were found only by some of the labs (Figure 3). The average percentage of discrepancies from the consensus was slightly higher with the HISTO SPOT® test and this was mainly due to a higher number of false negative reactions. However, both tests are far below the 25% of discrepancies that are "allowed" to pass the external proficiency testing (Figure 4).

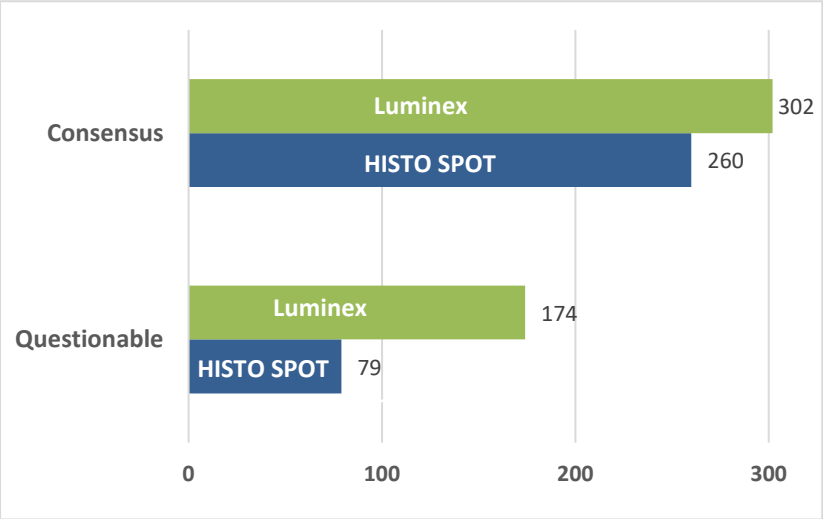


Figure 3: Number of consensus and questionable specificities found with the EPT 2019 sera using the Luminex test (67 labs) and the HISTO SPOT test (11 labs)

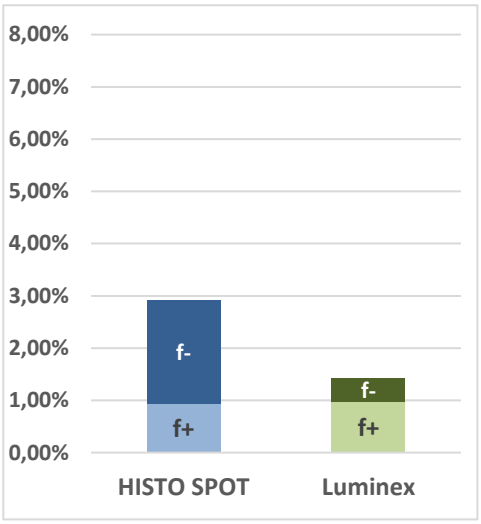


Figure 4: Average percentage of discrepancies per lab compared to the number of consensus specificities for the EPT 2019 sera

Semiquantitative analysis of results for EPT sera:

The average global CV for the signal (=MCI) was 48%, for the S/B ratio it was 28% and for the mean it was around 13%. The completely negative serum EPT 2019 F had a much higher global CV of the Mean than the other sera (Figure 5). The signal and the S/B ratio are both corrected for the background intensity, whereas the mean is the raw value. Obviously, the background correction contributes to the variability of the intensity values. The completely negative serum shows only background variation, and this seems to be higher than the variation of the positive reactions. The variation for the EPT sera is caused by different factors because they were generated in different labs, by different operators on different instruments and with four different batches.

There was considerable variation of the background which explains that the parameters Signal and S/B show a higher variability than the Mean parameter (see Figure 6). Serum EPT 2019 F shows an exceptionally high global CV for the background as well. Looking at one of the images for the serum (Figure 7) reveals that there is an unspecific background reaction with almost all the antigens on the chip, which might explain the unusual results for this serum.

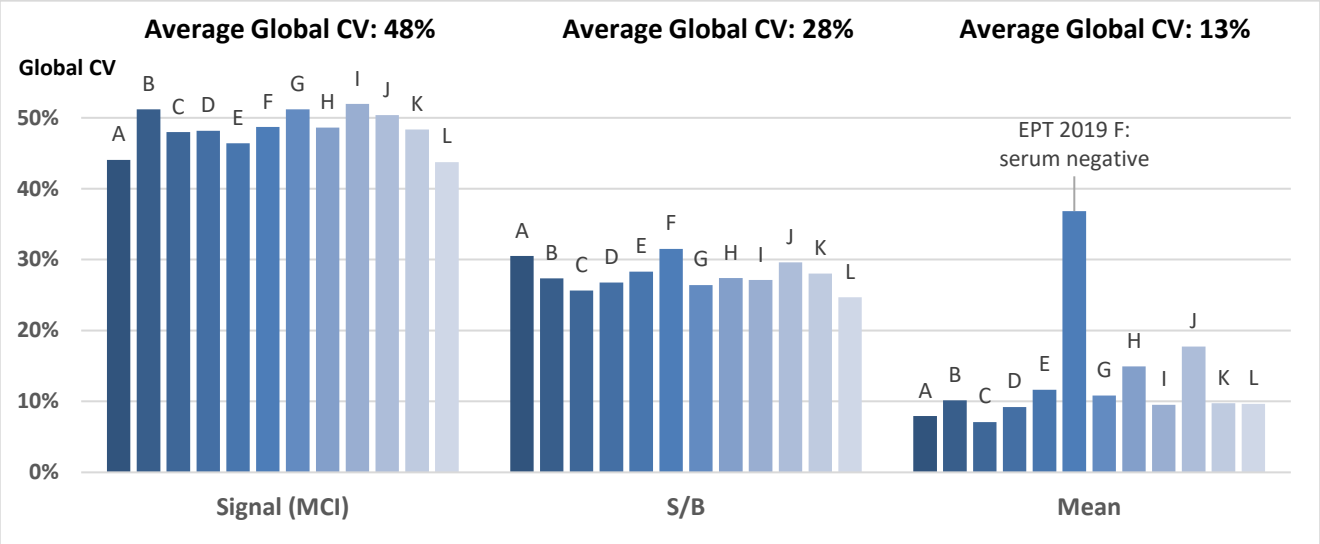


Figure 5: Global CV for 12 EPT 2019 sera tested in 11 labs
The global CV (mean CV of the 96 antigens on the chip) for each serum is shown for the different parameters used to measure the intensity of the reaction. Each bar in the histogram represents one serum. The average global CV is given as the mean of the CVs of the 12 sera.

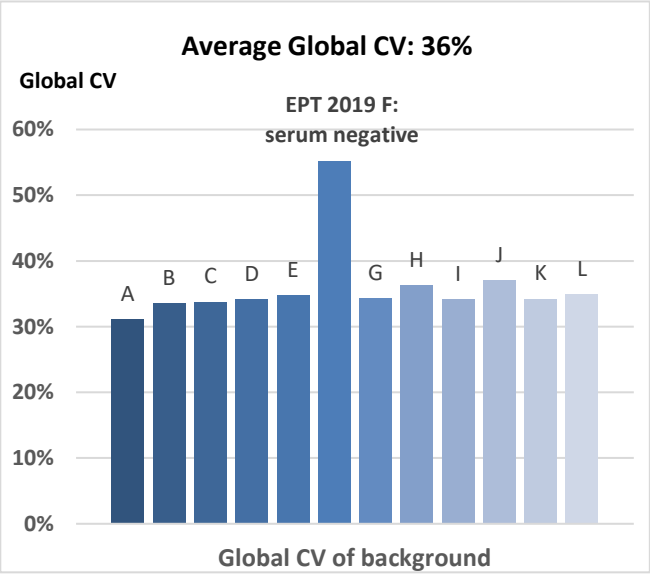


Figure 6: Global CV for the background in 12 EPT 2019 sera tested in 11 labs
The global CV (mean CV of the 96 antigens on the chip) for each serum is shown for the background variability. Each bar in the histogram represents one serumThe global CV (mean CV of the 96 antigens on the chip) for each serum is shown for the different parameters used to measure the intensity of the reaction. Each bar in the histogram represents one serum. The average global CV is given as the mean of the CVs of the 12 sera

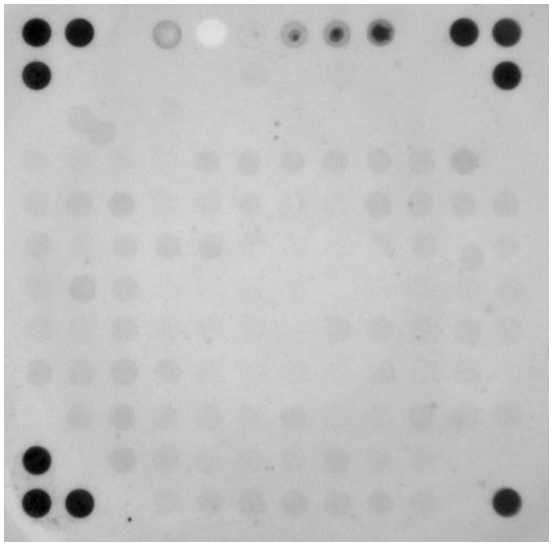
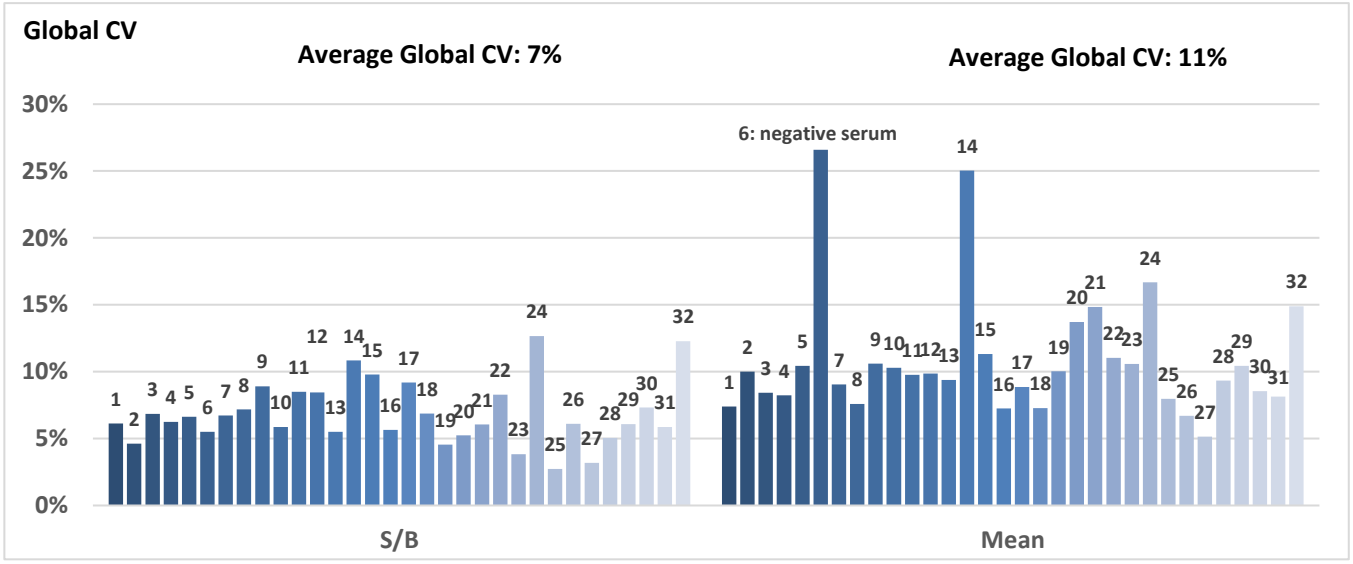


Figure 7: Array image for serum EPT 2019 F

Semiquantitative analysis for QC sera:

The analysis of the global CV for the background corrected signal was not possible because the mean for signal was zero for some of the antigens and the global CV could not be calculated. The batch to batch variability on one instrument was in the same range as for the EPT sera for the mean with 11% and it was lower for the S/B ratio with 7%. Again, one negative serum had an exceptionally high global CV for the mean value.

For the batch to batch variability the best reproducibility is achieved for the S/B ratio. The average global CV of the background was only 10 % and much lower than for the EPT sera (Figure 8). For two sera the background variation and the variation of the mean were exceptionally high. The reason for this might be unspecific background as has been seen with EPT 2019 F



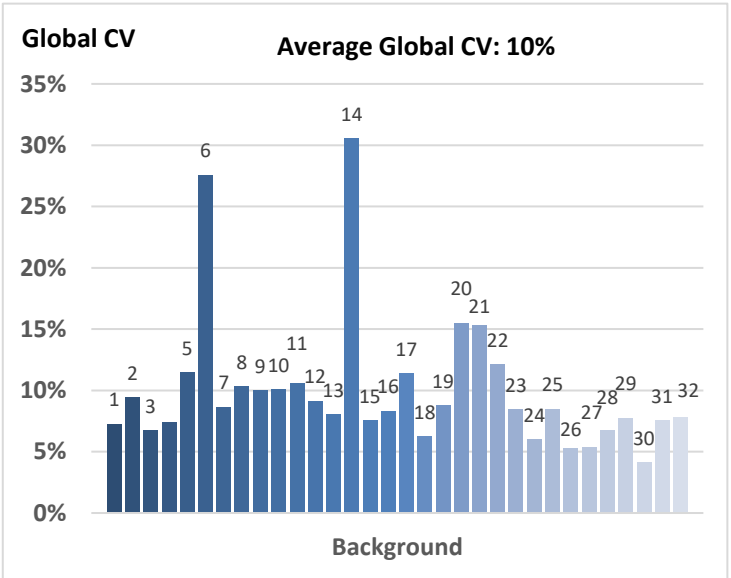


Figure 8: Global CV of the signal/background ratio (S/B), the mean and the background for 32 sera used in QC on the same instrument with 8 different batches (each bar represents one serum). The average global CV is given as the mean of the CVs of the 12 sera

Discussion

Concordance of qualitative results for EPT sera:

With an average of less than 3% discrepancies to the consensus result the concordance of results was good and far below the limit of 25% to pass the external proficiency testing. The percentage of discrepancies for the Luminex tests was only slightly lower. The questionable reactions as well as the false positive ones were generally weak reactions – this corresponds to Luminex results were the MFI is sometimes just a bit below or above the cut off.

The Luminex tests gave a higher number of consensus specificities but the clinical relevance of those is still debated (e.g. Roelen et al. 2012) and some of them might be due to artefacts like antibodies directed to denatured antigens or be "natural antibodies" (Pereira et al., 2011; El-Awar et al. 2009, Poli et al. 2011, Jacob et al. 2011, Carrie et al. 2016, Ravindranath et al. 2017). Therefore, the lower number of specificities found by the HISTO SPOT® test might reflect a higher specificity rather than a lower sensitivity compared to the Luminex tests. Further studies including direct evidence for the clinical relevance like crossmatches or transplantation outcome are required for a final conclusion.

Interestingly, the number of questionable results found by only some of the labs was twice the number found with HISTO SPOT® test when the 75% consensus applied for both tests. With the 96% consensus the number of questionable specificities would even be higher for the Luminex tests. This reflects a large grey area where results are sometimes positive and sometimes negative with the Luminex tests. Though the comparison is difficult because of the different number of participating labs and different consensus levels it can be concluded that the reproducibility of the HISTO SPOT® test is good and at least equivalent to the Luminex tests.

Semiquantitative results for EPT sera and QC sera:

For the EPT sera the mean intensity of the spot uncorrected for the background had the lowest global CV of 13% for the results from 11 different labs – and different instruments. In comparison the background variation was quite high with global CV of 36%. However, for the QC sera tested all on the same instrument the global CV was generally low and the lowest variation was observed for signal to background ratio. The global CV of the background was much lower as well. This might be explained by slightly different calibrations of the cameras in the MR.SPOT instruments leading to different standard grey values for the background. Another reason can be different serum treatment methods in different labs like heat treatment which can result in higher or lower background.

If the HISTO SPOT® test shall be used for monitoring of donor specific antibodies after transplantation where tests will be run on the same instrument, the best parameter to follow would, therefore, be the signal to background ratio. Using signal to background ratio for the lowest spot of the locus might as background value might reduce variability even more and perhaps a cut off based on this value can be defined (Wisse et al. 2019).

To compare intensities between labs, however, the most suitable parameter would be the Mean signal.

Compared to the global CV values for the MFIs reported for the Luminex tests in the literature the variation of the signals for the HISTO SPOT® tests are clearly lower (see Table 2).

Therefore, with regard to the variation of the signal intensity the HISTO SPOT® test shows a better consistency from batch to batch and from lab to lab.

Table 2: Global CV of signal intensities in different studies

Publication	Global CV class I	Global CV class II	Database	Remarks
Liu et al. 2015 Luminex	24%	21%	MFI adjusted for background for 1 mixed positive control serum, 20 runs	• Differences between 6 operators found • CV decreases with higher MFI
Locke et al. 2017 Luminex	30%	26%	36 sera from UCLA exchange tested in 9-16 labs, global CVs only for consensus specificities	
Wisse et al. 2019 Luminex	23%	18%	12 EPT sera from 2016 tested 3 labs with Luminex test from the same vendor	decreased CV when using signal to background ratio
Reed et al. 2013: Luminex	28% 32%	24% 22%	14/16 sera for class I / class II in different labs / one batch raw data manufacturer's standardized data	differences between two vendors, CV decreases with higher MFI
This study HISTO SPOT®	13% 28%	nt nt	12 EPT sera in 11 labs / 4 batches Mean: raw data Signal / background ratio	
This study HISTO SPOT®	11% 7%	nt nt	32 QC sera in 1 lab / 8 batches Mean: raw data Signal / background ratio	

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Morales-Buenrostro LE, Tearasaki PI, Marino-Vázques LA et al. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation* 2008; 86: 1111-1115

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BAG Diagnostics GmbH, Amtsgerichtsstr. 1-5, 35423 Lich / Germany

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