DETERMINATION OF SCREENING CUT POINT, SPECIFICITIY CUT POINT, AND SENSITIVITY OF AN ANTI-DRUG ANTIBODY BRIDGING ELISA

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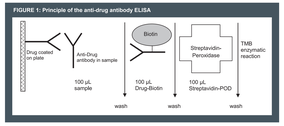
OBJECTIVES

A quasi-quantitative bridging ELISA was validated according to the principles described in the most recent recommendations for immunogenicity assay validation [1]. A major focus of this white paper is the application of statistical procedures to ensure objectivity.

METHODOLOGY

The drug (a therapeutic humanized monoclonal antibody) was coated onto the ELISA plates and human serum samples (minimal required dilution: 1:100) with affinity purified goat anti-drug antibodies were used as positive controls. The detection of captured antibodies was performed with biotinylated drug and streptavidin peroxidase followed by TMB/H_2O_2 enzymatic reaction.

FIGURE 1: Principle of the anti-drug antibody ELISA

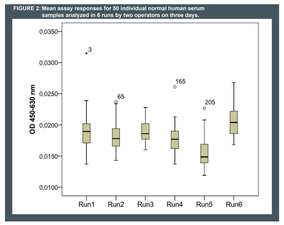


RESULTS

Determination of Screening Cut Point

The screening cut point is defined as the level of response of the screening assay at and above which a sample is considered to be reactive for the presence of antibodies. To establish the screening cut point, fifty individual normal human sera divided on three microplates were repeatedly analyzed on three days by operator 1 (Run1, 3, 5) and operator 2 (Run2, 4, 6).

FIGURE 2: Mean assay responses for 50 individual normal human serum samples analyzed in 6 runs by two operators on three days.



Group	Mean	Variance	CV%
Run 1	0.0186	5.44E-06	12.5%
Run 2	0.0180	4.23E-06	11.4%
Run 3	0.0189	2.30E-06	8.0%
Run 4	0.0176	3.64E-06	10.8%
Run 5	0.0154	3.81E-06	12.7%
Run 6	0.0206	6.39E-06	12.3%

Determination of validation cut point and correction factor

A Non-parametric method

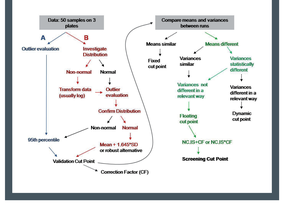
1 Outliers were identified using the boxplot method. Data points that were beyond the quartile by 1.5 times the interquartile range were classified as outliers.
2 The 95th percentile of the outlier-eliminated OD values was set as validation cut point in order to allow a rate of 5% false positives.
3 The correction factor (CF) was calculated by subtracting the mean of the validation negative controls (NCs) from the validation cut points.

B Parametric method

1 Data distribution was investigated using the Shapiro-Wilk test for normality. Non-normality was found for runs 1, 4, 5, and 6. Accordingly, log transformation was aplied.
2 After outlier determination using the boxplot method, data distribution was rechecked and confirmed as normal.
3 Mean + 1.645*SD was set as validation cut point in order to allow a rate of 5% false positives ($t_{0.05}$ = 1.645).
4 The correction factor (CF) was calculated by dividing the validation cut points by the mean of the validation negative controls (NC).

	Non-parametric method	Parametric method
Count	296	297
Mean OD of ind. samples	0.0182	-1.7441 (log transformed)
Validation cut point	**0.0217**	**0.0218**
Mean OD of NC	0.0176	0.0176
CF	**0.0041**	**1.2376**

FIGURE 3: Scheme for evaluating the screening cut point. A non-parametric (A) and a parametric (B) approach were used to determine validation cut point and correction factor. Statistical analysis of means and variances implicated that a run-specific floating cut point is most feasible. Finally, the run-specific screening cut point can be calculated using the run-specific negative control and the correction factor determined in validation.
NC:SI: negative control from in study sample. Modified from [1]



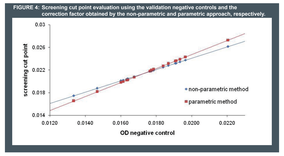
Calculation of screening cut point

Single factor ANOVA p-value of 2.71 * 10^{-27} suggested that run means are statistically different (at alpha = 0.05 significance level), not allowing the use of a fixed cut point.

The Levene's test showed that run variances are also statistically different (p-value 0.031). According to the recommendations given in [1], an instrument or analyst specific floating cut point or a dynamic cut point should be applied then. However, the absolute values for the variances are at very low levels (highest variance at 0.00000639), suggesting that the statistically significant differences are not different in a relevant way. Accordingly, a floating cut point was used.

Screening cut point (non-parametric) = NC + 0.0041
Screening cut point (parametric) = NC * 1.2376

FIGURE 4: Screening cut point evaluation using the validation negative controls and the correction factor obtained by the non-parametric and parametric approach, respectively.



Both non-parametric and parametric approach resulted in similar screening cut points as long as the in study negative control was in the expected region. For low and high negative controls, however, differences between the two approaches were found. Such differences in the floating screening cut point could potentially influence the rate of false positive samples. For the current example, the rate of false positives was 5% and 4% for non-parametric and parametric approach, respectively, nicely corresponding to the desired rate of false positives, which was set to 5%.

Determination of Specificity Cut Point

The specificity cut point is defined as the percentage inhibition at or above a sample is considered as "confirmed antibody positive". To establish the specificity cut point, 50 individual normal human sera divided on three microplates were repeatedly analyzed in the presence (inhibited sample) and absence (uninhibited sample) of drug on three days by two operators each. The response for the samples analyzed in the presence of drug was compared to the response in absence of drug.

% inhibition = 100 * [1- (drug inhibited sample / uninhibited sample)]

FIGURE 5: %inhibition for 50 individual normal human serum samples an alyzed in 6 runs.

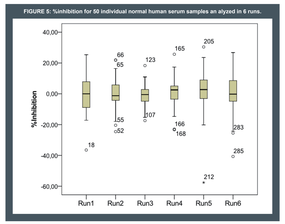
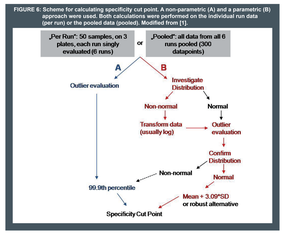


FIGURE 6: Scheme for calculating specificity cut point. A non-parametric (A) and a parametric (B) approach were used. Both calculations were performed on the individual run data (per run) or the pooled data (pooled). Modified from [1].



A Non-parametric method

1 Outliers were evaluated according to the boxplot method.
2 The 99.9th percentile of the outlier-eliminated data was set as specificity cut point in order to allow a rate of 0.1% false positives.
3 The actual rate of false positives among the 300 data points was calculated.

B Parametric method

1 The distribution was investigated using the Shapiro-Wilk test for normality. Non-normality was found for runs 5 and 6.
2 To apply log transformation, negative values have to be avoided and the %inhibition is not feasible. The ratio of drug inhibited to uninhibited samples was used instead.
3 After outlier determination using the boxplot method, the log transformed data was normally distributed.
4 Mean – 3.09*SD was used to calculate the specificity cut point in order to allow a rate of 0.1% false positives ($t_{0.001}$ = 3.09).
5 Data was back-transformed to %inhibition by calculating antilog and 100*(1-inhibited/uninhibited values).
6 The actual rate of false positives among the 300 data points was calculated.

	Non-parametric method		Parametric method	
	Per Run	Pooled	Per Run	Pooled
Count	284	285	288	284
Mean inhibition of ind. samples	1.2%	0.7%	-0.0040 (log transformed)	-0.0031 (log transformed)
Specificity cut point	**19.9%**	**21.9%**	**24.4%**	**23.6%**
False positives	**3.3%**	**2.3%**	**1.3%**	**1.7%**

Major differences in the specificity cut points calculated by the non-parametric and parametric approach were found, resulting in a higher rate of false positives using the non-parametric method. However, in both approaches, the rate of false positives was by far higher than the desired rate of false positives which was set to 0.1%. This might be caused by the high amount of outliers (about 5%) found by the boxplot method. Their elimination substantially decreases variance and, accordingly, the specificity cut point. Another method of outlier identification could possibly be more feasible for the determination of the specificity cut point.

Assay Sensitivity

Assay sensitivity for ADA assays is defined by the lowest concentration at which a positive control antibody preparation consistently provides a positive signal in the assay. Eight serial dilutions of a positive control containing 1000 ng/mL affinity purified goat anti-drug antibodies in human serum were tested in total 12 runs by two operators.

FIGURE 7: Representative dilution curve of the positive control goat anti-drug antibody. Dilution curves were fitted by the 4-PL curve fitting method and the corresponding concentrations at the run-specific screening cut point value (NC + 0.0041) were interpolated.

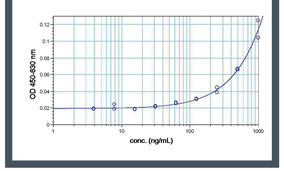
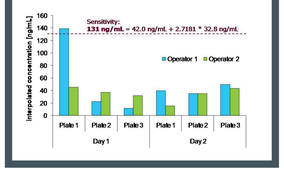


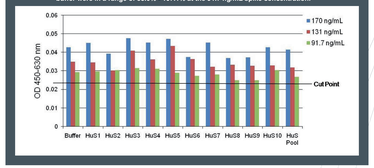
FIGURE 8: Interpolated antibody concentrations at the run-specific screening cut point (NC + 0.0041) compared to sensitivity calculated for a 95% consistency level (Sensitivity = Mean interpolated concentration at cut point + $t_{0.05}$ * SD). The high interpolated concentration on the first plate was due to an unexpectedly high OD value for the negative control.



Assay Sensitivity / Interferences by Matrix Components

The concentration corresponding to the assay sensitivity determined by antibody dilution curves (131 ng/mL), 70% of this concentration (91.7 ng/mL), and 130% (170 ng/mL) were spiked in assay buffer, in 10 normal individual human serum samples, and in human serum pool.

FIGURE 9: Recovery of low spike concentrations in human serum samples and assay buffer. All results were above the screening cut point and mean recoveries compared to assay buffer were in a range of 88.0% - 107.1% at the 91.7 ng/mL spike concentration.



CONCLUSION

Assay characteristics:

Analyte:	Human antibodies against a humanized therapeutic antibody (affinity purified goat anti-drug antibody used for controls)
Matrix:	Human serum
Min. Req. dilution:	1:100
Screening cut point:	Floating cut point (NC + 0.0041, NC * 1.2376) Target false positive rate: 5.0%
Observed false positives:	5.0% (4.0%)
Specificity cut point:	19.9% - 24.4% inhibition Target rate of falsely confirmed samples: 0.1% Observed falsely confirmed samples: 1.3% - 3.3%.
Assay sensitivity:	91.7 ng/mL

Statistical evaluations:

- The applied statistical procedure allowed an objective setting of the cut points.
- The Levene's test for equivalence of variance on the 95% confidence level could not confirm equivalence. The variances at that low level, however, are not considered to be different in a relevant way. Therefore, a floating cut point is considered as the most appropriate type though.
- The observed ratio of falsely confirmed positives exceeded the target range by more than factor 10 because of the outlier elimination in order to make the specificity cut point more conservative.

REFERENCES

[1] Gopi Shankar et al. J.Pharm. Biomed. Anal. 48 (2008) 1267-1281

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