ELISA Data Analysis

One of the major difficulties in ELISA tests can be the determination of the cut-off or threshold value, which discriminates positive results from background readings. The background or the reaction of healthy samples in ELISA-tests depends on a variety of different factors such as reagents, chemicals (purity of pNPP!), type of microtiter plate, incubation conditions, kind of plant tissue, but also on handling (especially washing!). Even if all these parameters are kept as constant as possible, it may happen that there are differences from plate to plate in the same test series. Therefore, it is not advisable to work with a fix OD value as cut-off, but to calculate it for each microtiter plate.

Any method of setting the cut-off of OD values is arbitrary. Here, we describe a statistical analysis that has proven its usefulness in our laboratory and can easily be calculated for each microtiter plate. We recommend to use a spreadsheet program such as MS-Excel allowing the programming of macros for the presented formulas and the sorting of data. For an automated processing of data, we use a fix design for the distribution of test samples in a 96-well microtiter plate. Below, an example is given for distributing 40 samples in duplicate wells, together with a positive and a negative control and a buffer blank (Fig. 1).

Fig. 1. Distribution of 40 duplicate samples in a 96-well ELISA plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Extraction buffer	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	positive
B		1	5	9	13	17	21	25	29	33	37	control
C		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	negative
D		2	6	10	14	18	22	26	30	34	38	control
E		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
F		3	7	11	15	19	23	27	31	35	39	
G		sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	
H		4	8	12	16	20	24	28	32	36	40	

Tab. 1. These raw data are a typical example of our test service for a nursery where we have tested pelargonium plants for the presence of the pathogen *Xanthomonas campestris* pv *pelargonii* (Xcp).

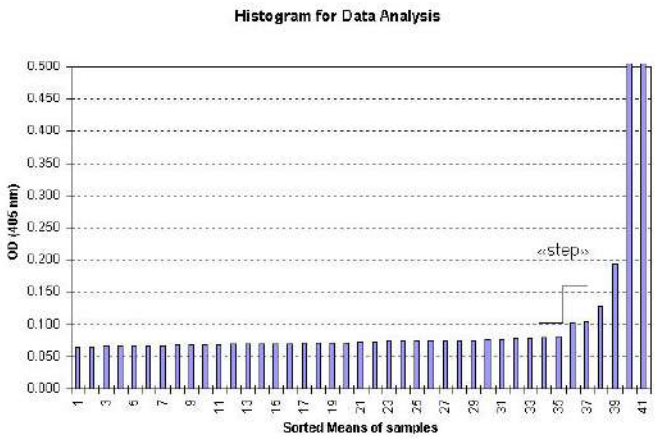
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.069	0.064	0.067	0.068	0.068	0.102	0.071	0.072	0.068	0.065	0.063	2.098
B	0.070	0.064	0.067	0.066	0.068	0.102	0.066	0.068	0.065	0.070	0.067	2.026
C	0.073	0.069	0.073	0.068	0.077	0.070	0.068	0.071	0.072	0.557	0.068	0.069
D	0.075	0.074	0.077	0.071	0.082	0.072	0.070	0.074	0.076	0.592	0.071	0.074
E	0.078	0.200	0.082	0.074	0.076	0.072	0.134	0.075	0.075	0.076	0.089	0.071
F	0.075	0.188	0.075	0.071	0.075	0.071	0.120	0.073	0.073	0.078	0.074	0.073
G	0.073	0.065	0.070	0.071	0.073	0.073	0.104	0.074	0.076	0.067	0.067	0.069
H	0.073	0.069	0.070	0.071	0.075	0.077	0.106	0.076	0.077	0.069	0.064	0.071

By eye, a quite even distribution of buffer and sample values of approx. 0.070 OD can be observed. Some of the values, however, are slightly increased, e.g. 6A/6B with OD values of 0.102. The following procedure allows to decide if this sample (sample 17) is positive i.e. can be distinguished from the background of «healthy» samples.

First, calculate the means of the two readings of each sample and sort these means in ascending order. By making a histogram, data belonging to the background can be recognized as a slight linear increase of OD values. A 'step' distinguishes potential positive samples (at least the positive control) from the preceding background values. In Fig. 2, an example is given with the data of Tab.1.

www.bioreba.com
BIOREBA AG
Christoph Merian-Ring 7
CH-4153 Reinach
Switzerland
Your Partner in Agro-Diagnostics
Phone +41 61 712 11 25
Fax +41 61 712 11 17
admin@bioreba.ch
www.bioreba.com

Fig. 2. Histogram of sorted mean values.



In this histogram, means 1 - 35 show a linear increase of OD values. The 36th value exhibits a clear increment compared to the foregoing values. This «step» has to be determined for each processed microtiter plate. The calculation of the cut-off then indicates if the next values on the right side of the step (indicated with an arrow) can be considered as positive or not; in other words, if these values are significantly different from the preceding «background». In this example, the calculation of the cut-off has been done with mean values 1-35 using the following formula (Tab. 2):

Tab. 2. Formula for calculation of the cut-off value

cut-off = (mean + 3s) x 1.1
mean: mean of the mean values up to the step (mean values 1-35) = 0.072 **s**: standard deviation of first 35 mean values = 0.004 **cut-off** = (0.072 + (3 x 0.004)) x 1.1 = 0.092

Tab. 3. Calculation of means and comparison with cut-off value (values in mOD)

	1	2	3	4	5	6	7	8	9	10	11	12
mean	**73**	**64**	**67**	**67**	**68**	**102**	**69**	**70**	**67**	**68**	**65**	**2062**
A		64	67	68	68	102	71	72	68	65	63	2098
B		64	67	66	68	102	66	68	65	70	67	2026
result		-	-	-	-	**positive**	-	-	-	-	-	**positive**
mean		**72**	**75**	**70**	**80**	**71**	**69**	**73**	**74**	**575**	**70**	**72**
C		69	73	68	77	70	68	71	72	557	68	69
D		74	77	71	82	72	70	74	76	592	71	74
result		-	-	-	-	-	-	-	-	**positive**	-	-
mean		**194**	**79**	**73**	**76**	**72**	**127**	**74**	**74**	**77**	**82**	**72**
E		200	82	74	76	72	134	75	75	76	89	71
F		188	75	71	75	71	120	73	73	78	74	73
result		**positive**	-	-	-	-	**positive**	-	-	-	-	-
mean		**67**	**70**	**71**	**74**	**75**	**105**	**75**	**77**	**68**	**66**	**70**
G		65	70	71	73	73	104	74	76	67	67	69
H		69	70	71	75	77	106	76	77	69	64	71
result		-	-	-	-	-	**positive**	-	-	-	-	-

In Tab. 3, all means were compared with the cut-off value of 92 mOD. The means of the samples 3 (194 mOD), 17 (102 mOD), 23 (127 mOD), 24 (105 mOD), and 34 (575 mOD) were interpreted as positive. To avoid false positives, check the single values as well. In some cases, one value can be relatively high (e.g. caused by insufficient washing) and therefore giving a mean that corresponds not to the reality. Therefore, as a rule, check if both values contributing to the calculation of the mean are above the cut-off.

Recommendation: The level of background can be different for different plant tissues (e.g. leaves, sprouts, and tubers) or for different species/varieties of plants. In order to obtain an even distribution of the unspecific reactions contributing to the background, use as homogenous samples as possible in one microtiter plate.

Conclusion: This data interpretation helps to avoid false negatives in case of weak positive samples with an antigen concentration near the detection limit. It allows therefore taking full advantage of the sensitivity of ELISA tests.