Validation Report

AlerTox ELISA Milk
KIT3041/KT-5918

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1. Scope

The AlerTox ELISA Milk is designed for the determination of milk protein in food. The present report describes the validation process and its results.

2. Precision

A) Intra-Assay Variation

The intra-assay variation was determined by testing three controls of various concentration levels in 20fold replicates.

Table 1: Intra-assay variation of the AlerTox ELISA Milk

Replicate	Level 1	Level 2	Level 3	
1	0.49	2.54	10.5	
2	0.50	2.51	8. 7	
3	0.54	2.54	8.7	
4	0.58	2.70	9.8	
5	0.51	2.93	8.8	
6	0.59	2.72	10.6	
7	0.45	2.53	9.8	
8	0.55	2.59	9.9	
9	0.51	2.62	9.4	
10	0.46	2.42	11.4	
11	0.58	2.76	11.1	
12	0.59	2.56	8.7	
13	0.58	2.50	11.3	
14	0.46	2.58	10.7	
15	0.47	2.69	11.3	
16	0.46	2.38	9.4	
17	0.50	2.60	10.5	
18	0.45	3.03	11.8	
19	0.55	3.13	10.0	
20	0.55	3.09	11.4	
Mean	0.52	2.67	10.2	
SD	0.05	0.22	1.0	**RMS**
CV [%]	**9.8**	**8.1**	**10.0**	**9.3**

The coefficient of variation is ranging from 8.1% to 10.0% depending on the concentration.

RMS = Root Mean Square

B) Inter-Assay Variation

The inter-assay variation was determined by testing three controls of various concentration levels in four different test runs of the same kit lot.

Table 2: Inter-assay variation of the AlerTox ELISA Milk

Assay No.	Level 1	Level 2	Level 3	
1	0.54	1.95	6.5	
2	0.49	1.82	7.4	
3	0.47	1.81	5.6	
4	0.58	2.29	8.5	
Mean	0.52	1.97	7.0	
SD	0.05	0.22	1.25	RMS
CV [%]	9.7	11.4	17.8	13.4

The coefficient of variation is ranging from 9.7% to 17.8% depending on the concentration.

3. Recovery

For recovery experiments different sample matrices were spiked with milk protein to obtain various final concentrations after performing all sample pre-treatment steps. Tested samples and results were as follows.

Table 3: Recovery of various samples tested with the AlerTox ELISA Milk

Cookies

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.45	91
2 ppm	2.27	113
8 ppm	8.10	101
	Mean	**102**

Soy milk

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.42	84
2 ppm	1.63	82
8 ppm	5.84	73
	Mean	**79**

Sausage

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.50	99
2 ppm	1.64	82
8 ppm	6.62	83
	Mean	**88**

White wine

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.61	121
2 ppm	2.50	125
8 ppm	9.51	119
	Mean	**122**

Bread crumbs

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.56	111
2 ppm	2.30	115
8 ppm	8.23	103
	Mean	**110**

Orange juice

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.55	110
2 ppm	1.88	94
8 ppm	9.08	114
	Mean	**106**

Chocolate (based on soy)

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.49	99
2 ppm	2.21	110
8 ppm	6.96	87
	Mean	**99**

Cacao

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.18	36
2 ppm	0.46	23
8 ppm	1.68	21
	Mean	**27**

Cacao + 1 g Extraction Additive / 0.5 g Sample

Target Value	Actual Concentration	Recovery [%]
0.5 ppm	0.50	100
2 ppm	2.40	120
8 ppm	6.51	81
	Mean	**100**

Mean recoveries are ranging from 79% to 122% depending on the sample matrix.

The following concentrations were determined for the un-spiked samples:

Sample	[ppm]
Cookies	0.00
Soy milk	0.00
Sausage	0.02
White wine	0.02
Bread crumbs	0.11
Orange juice	0.01
Chocolate	0.10
Cocoa	0.01
Cocoa + Additive	0.02

4. Analytical Sensitivity

For determination of the analytical sensitivity sample diluent and milk protein free matrix samples respectively were assayed in 24fold replicates. After identification of possible outliers the OD mean and standard deviation were calculated. The corresponding concentration of the OD mean + 3x standard deviation was defined as limit of detection. This results in limits of detection according to the following table:

Table 4: Matrix-dependent and matrix-independent analytical sensitivity of the AlerTox ELISA Milk

Replicate	Sample diluent [OD]	Soy Milk [OD]	Orange Juice [OD]	White Wine [OD]	Bread Crumbs [OD]	Cookies [OD]	Chocolate [OD]	Sausage [OD]
1	0.375	0.371	0.355	0.319	0.410	0.452	0.376	0.500
2	0.352	0.357	0.328	0.325	0.392	0.451	0.378	0.555
3	0.363	0.380	0.377	0.341	0.398	0.447	0.389	outlier
4	0.352	0.362	0.319	0.386	0.402	0.444	0.388	0.503
5	0.345	0.335	0.336	0.331	0.394	0.490	0.392	0.523
6	0.337	0.416	0.407	0.348	0.414	0.460	0.369	0.554
7	0.336	0.346	0.452	0.314	0.399	0.470	0.367	0.474
8	0.419	0.335	0.351	0.344	0.451	0.483	0.379	0.473
9	0.345	0.320	0.327	0.304	0.377	0.437	0.367	0.479
10	0.347	0.334	0.353	0.329	0.368	0.458	0.369	0.468
11	0.371	0.392	0.352	0.351	0.370	0.465	0.429	0.494
12	0.332	0.350	0.344	0.338	0.386	0.462	0.350	0.525
13	0.343	0.452	0.335	0.334	0.397	0.484	0.381	0.539
14	0.345	0.441	0.422	0.351	0.395	0.452	0.365	0.488
15	0.367	0.325	0.337	0.334	0.385	0.486	0.353	0.500
16	0.384	0.341	0.317	0.334	0.394	0.463	0.373	0.483
17	0.357	0.315	0.321	0.331	0.367	0.453	0.350	0.449
18	0.349	0.330	0.327	0.335	0.367	0.447	0.362	0.443
19	0.372	0.399	0.358	0.347	0.368	0.452	0.361	0.588
20	0.333	0.333	0.343	0.321	0.406	0.466	0.361	0.462
21	0.376	0.475	0.330	0.329	0.401	0.487	0.419	0.489
22	0.369	0.319	0.420	0.336	0.387	0.450	0.361	0.650
23	0.345	0.356	0.331	0.321	0.382	0.456	0.365	0.470
24	0.390	0.343	0.345	0.334	0.385	0.474	0.352	0.451
Mean	0.359	0.364	0.354	0.335	0.391	0.462	0.373	0.503
SD	0.021	0.044	0.036	0.016	0.019	0.015	0.020	0.049
LOD [ppm]	**0.05**	**0.13**	**0.10**	**0.03**	**0.08**	**0.16**	**0.10**	**0.18**

With respect to the sample matrix limits of detection vary from 0.05 to 0.18 ppm. Note that the derived limits of detection are strictly dependent on the coefficient of variation and may thus vary in every individual test. The data for sample diluent and matrices respectively were not determined in the same test runs.

The lowest positive standard (0.5 ppm) was defined as limit of quantification to assure that all important matrices result in concentrations lower than this value.

5. Linearity

Linearity was determined by spiking different samples with milk protein and testing subsequent dilutions of the resulting extracts. For calculation of the linearity the highest concentration was defined as reference value (100%) and further dilutions were expressed in per cent of this reference after consideration of the dilution factor.

Table 5: Matrix dependent linearity of the AlerTox ELISA Milk

Soy milk

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	7.10	100
4 ppm	4.11	116
2 ppm	2.40	135
1 ppm	1.13	128
0.5 ppm	0.54	121
	Mean	**125**

Sausage

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	6.62	100
4 ppm	3.99	120
2 ppm	1.47	89
1 ppm	0.88	106
0.5 ppm	0.41	100
	Mean	**104**

White wine

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	6.71	100
4 ppm	3.70	110
2 ppm	2.16	129
1 ppm	1.06	126
0.5 ppm	0.55	131
	Mean	**124**

Orange juice

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	8.82	100
4 ppm	4.92	112
2 ppm	2.47	112
1 ppm	1.39	126
0.5 ppm	0.64	115
	Mean	**116**

Cookies

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	9.44	100
4 ppm	3.76	80
2 ppm	2.08	88
1 ppm	0.83	70
0.5 ppm	0.48	81
	Mean	**80**

Bread crumbs

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	6.16	100
4 ppm	3.88	126
2 ppm	2.13	138
1 ppm	0.95	123
0.5 ppm	0.49	127
	Mean	**129**

Chocolate (based on soy)

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	6.96	100
4 ppm	3.76	108
2 ppm	2.23	128
1 ppm	1.23	141
0.5 ppm	0.62	142
	Mean	**130**

Cocoa + *Extraction Additive*

Target Value	Concentration [ppm]	Recovery [%]
8 ppm	6.51	100
4 ppm	3.99	122
2 ppm	1.92	118
1 ppm	1.08	133
0.5 ppm	0.51	126
	Mean	**125**

For different matrices the mean linearity is ranging from 80% to 130%. The linearity is independent of the specific concentration and may only be affected by the intra-assay and inter-assay variation.

6. Cross-Reactivity

For the following foods no cross-reactivity (results < LOQ) were detected:

Table 6:   Non-cross-reactive food matrices in the AlerTox ELISA Milk

Food	c [ppm]
Pistachio	0.15
Peanut	0.10
Oats	0.05
Soy	0.04
Corn	0.05
Lupin	0.14
Barley	0.29
Walnut	0.29
Egg	0.12
Cocoa	0.07
Almond	0.09
Wheat	0.06
Celery	0.05
Hazelnut	0.06
Rice	0.32
Sesame	0.13
Beef (diluted 1+3)	0.16
Mustard	0.00
Cod	0.16
Shrimps	0.21
Pork (diluted 1+3)	0.20

The following cross-reactivities were determined:

Table 7:   Cross-reactive food matrices in the AlerTox ELISA Milk

Food	Cross-reactivity [%]
Caseinate	98
ß-Lactoglobulin	88
Non-fat milk powder (NIST 1549)	35
Whole milk powder (NIST 8435)	23
Ewe's milk	0.94
Goat's milk	0.014

7. Robustness

Robustness was determined by variation of different handling parameters as defined in the instruction manual. The results were compared with the results of samples analyzed according to the intended method. An un-spiked cookie sample and a sample spiked with 1.5 ppm (variation of temperature / time) or 2 ppm (drift) of milk protein were analyzed respectively.

A) Variation of extraction temperature

The extraction temperature, defined as 60 °C, was changed to 25 °C, 50 °C and 70 °C, respectively.

Table 8: Variation of extraction temperature in the AlerTox ELISA Milk

Sample	Result 60 °C	Result 25 °C	Result 50 °C	Result 70 °C
Cookie 0 ppm	0,00	0,00	0,01	0,00
Cookie 1.5 ppm	1.56	1.13	1.14	1.42

The results show that the extraction temperature should not be below 60°C.

B) Variation of extraction time

The extraction time, defined as 15 min, was changed to 5 min, 10 min and 20 min, respectively.

Table 9: Variation of extraction time in the AlerTox ELISA Milk

Sample	Result 15 min	Result 10 min	Result 5 min	Result 30 min
Cookie 0 ppm	0,00	0,01	0,00	0,02
Cookie 1.5 ppm	1.48	1.17	1.14	1.74

The results show that the extraction time should not be below 15 min.

C) Drift

In contrast to the test procedure as defined in the instruction manual the incubation time of the samples was extended and reduced by 5 minutes compared to the calibrators (20 min).

Table 10: Drift in the AlerTox ELISA Milk

Sample	Result 20 min	Result 15 min	Result 25min
Cookie 0 ppm	0.00 ppm	0.00 ppm	0.01 ppm
Cookie 2 ppm	1.84 ppm	1.38 ppm	2.74 ppm

The results differ significantly. Drift in extensive test runs should be avoided by pipetting calibrators once before the samples and once after the samples, using the mean value for calculation.

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