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NordVal International Certificate

Issued for:	**BRT Inhibitor Test**
NordVal No:	051
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BRT Inhibitor Test

Manufactured and supplied by:

Analytik in Milch Produktions- und Vertriebs-GmbH
Kaiser-Ludwig-Platz 2
80336 München
Germany

fulfils the validation requirements of the NordVal Validation Protocol 2. The BRT Inhibitor Test is a brilliant black reduction test for detection of antibiotic residues.

The method is tested for penicillins, cephalosporins, macrolides, sulfonamides, tetracyclines, aminoglycosides and Chloramphenicol in raw bovine milk on microtiter plates. Detection capability for clear results and presumptive results of photometric and visual reading is stated. In order for the method to be applicable in the EU, the detection capabilities for the substances of interest must be below given EU Maximum Residue Limits (MRL). The detection capabilities and associated MRLs for the substances tested are given in Table 2. Examples of the probability of detection (POD) / dose-response curves are given in the certificate for illustration. POD curves for all substances are given in the validation report, which is enclosed as an annex.

The performance of the method was tested in a comprehensive validation study carried out at the laboratory of Milchprüfring Bayern e. V. and tested in a large proficiency test.

The production of BRT Inhibitor Test is ISO 9001:2015-accredited by LGA InterCert GmbH.

Date: 21 February 2025

Yours sincerely,

Hrólfur Sigurðsson

Hrólfur Sigurðsson
Chair of NordVal International

Eystein Oveland
NMKL Executive Director

PRINCIPLES OF THE METHOD

The BRT Inhibitor Test is a Brilliant Black Reduction test (BRT) containing the test bacteria G. *stearothermophilus* var. *calidolactis* C953, the redox indicator brilliant black, nutrients and other supplements. Antibiotic residues present in a sample can inhibit the growth of the test bacteria, thus preventing or decelerating the reduction of the colour indicator brilliant black and the consecutive colour change of the test medium from blue to yellow.

Milk samples (100 µl milk volume), positive and negative controls are added to the plates. Incubation is performed at 65 ℃ for 2 h 15 min ± 15 min until the complete discoloration of the negative control (colour change from blue to yellow) indicates the ideal reading time. Thereafter, milk is rinsed off the cavities for easier detection. The plates can be assessed with 2 different reading methods: visual examination or photometric evaluation using the measuring wavelength of 450 nm and the reference wavelength of 620 nm.

The recorded absorption values of the analysed samples are converted into relative percentage values by setting the average absorption level of the negative controls (yellow colour after incubation) as 0 % and that of the positive controls (blue colour after incubation) as 100 %, the absorption levels of the samples are set in relation to negative (0 %) and positive (100 %) controls.

The conversion formula is as follows: (S-NC)/(PC-NC) x 100 = X %
where

S	is the analysed sample´s absorption level
NC	is the average of the four negative controls' absorption levels
PC	is the average of the four positive controls' absorption levels
X	is the relative percentage value of the analysed sample

Results are referred to as either Class A or Class B as shown in **Table 1**.

Table 1. Relation of reading systems and classes of results.

	Classes of results		
Reading system	Positive Class A Clear	Positive Class B Presumptive	Negative
Visual	1	2	0
Photometric	≥ 65 %	40 % - < 65 %	< 40 %

SAMPLES USED IN THE STUDY

High-quality raw ex-farm bulk milk proven free of antibiotic residues was spiked with antibiotics. To verify the correct concentration of the stock solutions and the spiked raw milk samples, serial dilutions of the prepared positive samples were analysed with microbiological inhibitor and, when applicable, with receptor tests or analysed by LC-MS/MS.

Batch-to-batch variation

Potential deviations in the detection capabilities of different plate batches were evaluated statistically, and it was shown that there were no statistically significant differences between the batches of plates.

METHOD PERFORMANCE CHARACTERISTICS

Selectivity

Marker substances of commonly used classes of veterinary drugs other than antibiotics were analysed with photometric reading in order to determine the selectivity of the BRT Inhibitor Test. The investigated compounds included the anti-inflammatories Flunixin, Metamizole (NSAIDs) and Prednisolone (glucocorticoid) as well as antiparasitic substances (Triclabendazole and Deltamethrin). Furthermore, the polyether-antibiotic Monensin, used for ketosis treatment in dairy cows, was tested. The substances were spiked at a concentration of 100 x EU MRL and inoculated with 6 replicates. None of the tested substances inhibited the growth of the test germs, leading to negative results (both Class A and Class B). Thus, no false positive results were observed.

Detection capability (CCβ)

For the determination of the detection capability (CCβ), three different batches of plates were used at all times. The lowest concentration obtaining a minimum of 95 % positive results was considered as detection limit (CCß). Based on the different interpretation methods, CCß A and CCß B (for Class A results: clear; and Class B results: presumptive, respectively - Table 1) were established in parallel for each substance. Different substances were tested. Table 2 shows the results. The substances exceeding the EU MRLs or having high detection capability (Chloramphenicol) are marked in red and written in italic.

In the EU legislation, maximum residue limits (MRLs) are stated for milk, concentrations of veterinary drugs below these limits are accepted. According to this, the BRT Inhibitor Test plates are applicable for the detection of the penicillin compounds: Benzylpenicillin, Ampicillin, Amoxicillin, Cloxacillin, Dicloxacillin, Nafcillin and Oxacillin, for the cephalosporins: Cefapirin, Cefoperazone, Cefazolin and Cefalonium; and for Neomycin. The method was found not to be applicable for the detection of macrolides, sulfonamides, tetracyclines and aminoglycosides, other than Neomycin, at the maximum permitted EU residue level (MRL). Further, for chloramphenicol, the EU minimum required performance limit (MPRL) is 0.3 µg/kg and hence the method is not applicable for this substance (Commission Decision of 13 March 2003 amending Decision 2002/657/EC).

Table 2. Substances tested, EU Maximum Residue Limit (MRL) and detection limits by

photometric and visual reading for CCβ A (>65 %) and CCβ B (40-65 %).

Group of antibiotics	Substance	MRL EU* [µg/kg]	CCβ A [µg/kg]	CCβ B [µg/kg]	CCβ A [µg/kg]	CCβ B [µg/kg]
			Photometric		Visual	
Penicillins	Benzylpenicillin	4	2.5	2	2.5	2
	Ampicillin	4	3.5	3	3.5	3
	Amoxicillin	4	3	2.5	3.5	3
	Cloxacillin	30	25	20	30	25
	Dicloxacillin	30	15	12.5	15	12.5
	Nafcillin	30	15	10	15	8
	Oxacillin	30	10	8	12	10
Cephalosporins	Cefalexin	100	400	300	500	400
	Cefapirin	60	6	5	8	5
	Cefoperazone	50	35	25	35	25
	Cefazolin	50	9	7	9	7
	Cefquinome	20	500	300	600	300
	Ceftiofur	100	200	150	250	200
	Cefalonium	20	14	12	16	12
Macrolides	Erythromycin	40	100	50	100	60
	Tylosin	50	75	40	100	30
Sulfonamides	Sulfadiazine	100	>800	100	>800	100
	Sulfadimethoxin	100	>800	200	>800	200
	Sulfamethazine	100	1,000	300	>1,000	200
	Sulfathiazol	100	400	60	400	60
	Sulfadoxin	100	>1,500	400	>1,500	400
	Sulfamethoxypyridazine	100	500	100	500	100
Tetracyclines	Chlortetracycline	100	>1,000	800	>1,000	800
	Oxytetracycline	100	800	400	800	400
	Tetracycline	100	1,000	600	1,000	600
Aminoglycosides	Dihydrostreptomycin	200	600	400	500	400
	Streptomycin	200	1,500	600	1,500	600
	Gentamicin	100	200	100	150	80
	Neomycin	1,500	400	200	300	200
Fenicol	Chloramphenicol	-	7,000	4,000	5,000	3,500

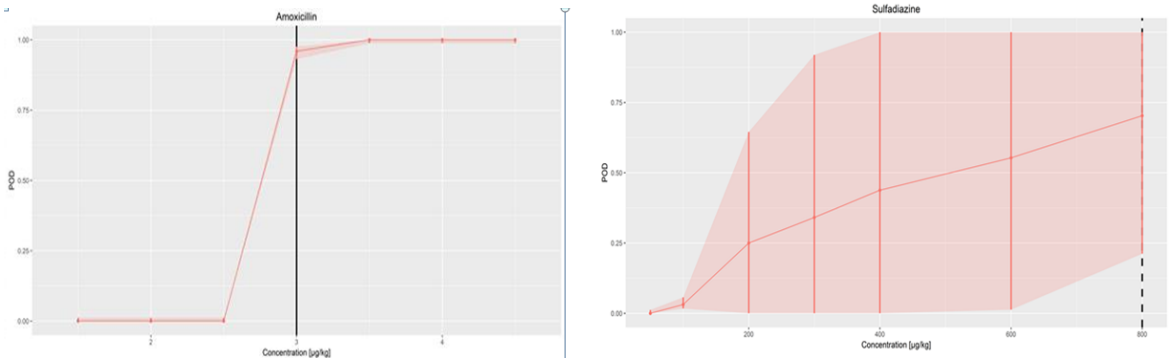
*Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.

Probability of detection (POD) / dose-response

Probability of detection was established based on the Class A results with both photometric and visual evaluation (results are presented in the validation report, Annex 1, Table 2). For combined photometric and visual readings of the class A results, lower and upper CIs were calculated (presented in the validation report, Figure 3 and in its annex, Figure 1).

Bactericidal substances like beta-lactams mostly exhibited steeply increasing dose-response curves and narrow CIs (example **Figure 1a**, **Table 3**). In contrast, bacteriostatic substances displayed more consistent curve increments and often showed bigger variations between the two reading methods (example **Figure 1b**, **Table 4**). The width of the confidence interval provides information about the uncertainty of the measurement as the wider the confidence interval the greater the uncertainty. Compared to photometric reading, the visual interpretation leads to bigger variances in the results and thus to wider CIs.

Figure 1. Dose-response curves for Amoxicillin (a) and Sulfadiazine (b), combined results for photometric and visual reading.



In **Table 3**, showing results of the dose-response study for Amoxicillin, it can be seen that at 2.5 µg/kg for photometric reading none of the samples were clearly positive (absorbance level ≥ 65 %), but 98 % of the results were presumptive positive having absorbance levels of 40 % - < 65 %.

For visual reading at the same concentration, none of the samples were clearly positive (Class A), but 74 % of the samples were presumptive (Class B). At 3.0 µg/kg, photometric reading gave 100 % clear positive results (Class A) and thus, the same (100 %) when clear positive and presumptive results were combined (CCβ B). At the same concentration for visual reading, 13 results were presumptive positive and adding this to the clear positive results (179 samples; 0.93 %) a total of 100 % CCβ B was obtained. At 3.5 µg/kg all samples were detected clear positive with visual reading.

Table 3. Dose-response for Amoxicillin

Reading system	Conc µg/kg	No. of total results	No. of results (pos. Class A)	Share of CCβ A	Lower 95%-CI (CCβ A)	Upper 95%-CI (CCβ A)	No. of results (pos. Class B)	Share of CCβ B	No. of results (negative)
Photo	1.5	128	0	0.00	-	-	0	0.00	128
	2	128	0	0.00	-	-	6	0.05	122
	2.5	128	0	0.00	-	-	126	0.98	2
	3	128	128	1.00	-	-	0	1.00	0
	3.5	128	128	1.00	-	-	0	1.00	0
	4	128	128	1.00	-	-	0	1.00	0
	4.5	128	128	1.00	-	-	0	1.00	0
Visual	1.5	192	0	0.00	-	-	0	0.00	192
	2	192	0	0.00	-	-	0	0.00	192
	2.5	192	0	0.00	-	-	142	0.74	50
	3	192	179	0.93	-	-	13	1.00	0
	3.5	192	192	1.00	-	-	0	1.00	0
	4	192	192	1.00	-	-	0	1.00	0
	4.5	192	192	1.00	-	-	0	1.00	0
Photo + visual	1.5	320	0	0.00	0.00	0.01	0	-	320
	2	320	0	0.00	0.00	0.01	6	-	314
	2.5	320	0	0.00	0.00	0.01	268	-	52
	3	320	307	0.96	0.93	0.98	13	-	0
	3.5	320	320	1.00	0.99	1.00	0	-	0
	4	320	320	1.00	0.99	1.00	0	-	0
	4.5	320	320	1.00	0.99	1.00	0	-	0

In **Table 4**, showing results of the dose-response study for Sulfadiazine, it can be seen that even at the highest concentration tested (800 µg/kg), the ratio of samples detected as clearly positive (absorbance level ≥ 65 %; Class A results) did not reach more than 74 % for photometric reading and 68 % for visual reading.

The sum of the presumptive and clear positive results (positive Class A and positive Class B) gives 100 % CCß B at 200 µg/kg (CCβ B) for both photometric and visual reading.

Table 4. Dose-response for Sulfadiazine.

Reading system	Conc µg/kg	No. of total results	No. of results (pos. Class A)	Share of CCβ A	Lower 95%-CI (CCβ A)	Upper 95%-CI (CCβ A)	No. of results (pos. Class B)	Share of CCβ B	No. of results (negative)
Photo	50	128	0	0.00	-	-	12	0.09	116
	100	128	0	0.00	-	-	123	0.96	5
	200	128	3	0.02	-	-	125	1.00	0
	300	128	0	0.00	-	-	128	1.00	0
	400	128	12	0.09	-	-	116	1.00	0
	600	128	49	0.38	-	-	79	1.00	0
	800	128	95	0.74	-	-	33	1.00	0
Visual	50	192	0	0.00	-	-	44	0.23	148
	100	192	10	0.05	-	-	176	0.97	6
	200	192	77	0.40	-	-	115	1.00	0
	300	192	109	0.57	-	-	83	1.00	0
	400	192	128	0.67	-	-	64	1.00	0
	600	192	128	0.67	-	-	64	1.00	0
	800	192	130	0.68	-	-	62	1.00	0
Photo + visual	50	320	0	0.00	0.00	0.01	56	-	264
	100	320	10	0.03	0.02	0.06	299	-	11
	200	320	80	0.25	0.00	0.65	240	-	0
	300	320	109	0.34	0.00	0.92	211	-	0
	400	320	140	0.44	0.00	1.00	180	-	0
	600	320	177	0.55	0.01	1.00	143	-	0
	800	320	225	0.70	0.21	1.00	95	-	0

Specificity - Rate of positive results not caused by residues of veterinary drugs

In order to demonstrate that the BRT Inhibitor Test performs properly with a broad range of samples, 704 ex-farm bulk milk samples were examined. Two out of 704 samples (0.28 %) were detected positive by the BRT Inhibitor Test. Both samples were confirmed to contain Cloxacillin. Thus, the rate of positive results not caused by residues of veterinary drugs was 0 %, as all positive samples detected were confirmed to contain antibiotic inhibitors (**Table 5**).

Table 5. The specificity tested in 704 samples.

	Negative samples		Positive samples			
Total no. samples	No.	Rate	No.	Rate	False positive	Con-firmed positive
704	702	99.72 %	2	0.28 %	0 %	100 %

False-negative rate (FN) / False-positive rate (FP)

In this validation, 154 test plates were analysed, including 616 positive control samples and 3,080 samples of negative raw milk, in total giving 3,080 (positive control) and 15,400 (negative milk) readings, respectively, with combined photometric evaluation and visual reading.

No false-positive results were observed when analysing the results of the negative milk samples by photometric evaluation (**Table 6**). One sample was classified false-negative by 1 out of 3 persons performing visual reading, whereas none of the other readers (technicians or photometric) assessed this sample as positive, the negative result is considered as outlier and might have been caused by a typing error.

Table 6. Rates of false-negative and false-positive results of the 3,080 positive controls and 15,400 negative milk samples.

	Rate of false results [%]				
Type of milk sample	ELISA 1	ELISA 2	Visual 1	Visual 2	Visual 3
Positive control	0	0	0	0	0.2
Negative control milk	0	0	0	0	0

The maximum relative percentage value obtained with photometric reading for negative samples was 33 %, whereas the minimum relative percentage value for positive samples was 93 %.

These values demonstrate that with the chosen thresholds for photometric reading (65 % Class A; 40 % Class B, Table 1) the false interpretation of positive as well as negative samples can be avoided.

INTERLABORATORY STUDY

The BRT Inhibitor Test was validated in an international proficiency test. 61 laboratories belonging to 55 companies originating from 10 countries participated in the examination of BRT Inhibitor Test plates provided for the interlaboratory study.

15 randomised and coded lyophilised UHT-milk samples were analysed - 8 samples contained antibiotics at MRL level, 7 samples consisted of inhibitor-free milk (**Table 7**), giving a total of 945 results. No false-positive or false-negative results were observed.

Table 7. The antibiotics analysed by 61 laboratories and the percentage of correct and false results.

Antibiotic	MRL (µg/kg)	No. samples	BRT Inhibitor test	
			Correct result (%)	False result (%)
Benzylpenicillin	4	2	100	0
Ampicillin	4	2	100	0
Cefapirin	60	2	100	0
Cloxacillin	30	2	100	0
Inhibitor-free milk	-	7	100	0

CONCLUSION

The BRT Inhibitor Test (microtiter plate format) was tested for detection of antibiotic residues in milk. For use and interpretation of the results the MRLs of the respective countries must be considered. The method is only applicable for substances with detection capability below the MRL or MPRL (where no MRL exists).

ANNEX 1

Validation Report BRT Inhibitor Test.