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NordVal International Certificate

Issued for:	**BRT MRL Screening Test**
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BRT MRL Screening Test

Manufactured by:

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fulfils the validation requirements of the NordVal Validation Protocol 2. The BRT MRL Screening Test is a Brilliant Black Reduction Test for the 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 MRL Screening Test is ISO 9001:2015-accredited by LGA InterCert GmbH.

Date: 21 February 2025

Yours sincerely,

Hrólfur Sigurðsson
Chair of NordVal International

Eystein Oveland
NMKL Executive Director

PRINCIPLES OF THE METHOD

The BRT MRL Screening Test is a modified 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 30 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 tests 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 MRL Screening 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 (CCβ). Based on the different interpretation methods, CCβ A and CCβ B (for Class A results: clear; and Class B results: presumptive, respectively - **Table 1**) were established in parallel for each substance. **Table 2** shows the results. The substances exceeding the EU MRLs or having a high detection limit (Chloramphenicol) are marked in red and written in italic.

In the EU legislation maximum residue limits (MRL) are stated for milk, and concentrations of veterinary drugs below these limits are accepted. According to this, the BRT MRL Screening 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 certain cephalosporins, macrolides, sulfonamides, tetracyclines and aminoglycosides at the maximum permitted EU residue level (MRL). Further, for chloramphenicol, the EU minimum required performance limit (MPRL) is 0.3 ug/kg and hence the method is not applicable for this substance (Commission Decision of 13 March 2003 amending Decision 2002/657/EC).

Some compounds could be detected at CCβ B level (presumptive results; 40 - <65 %, **Table 1**) only. Thus, for both photometric and visual reading presumptive results can be obtained for Ceftiofur, Tylosin, Sulfadiazine, Sulfadimethoxin, Sulfathiazol, Sulfamethoxypyridazine and Gentamicin.

Table 2. Substances tested, EU Maximum Residue Limits (MRL) and detection limits by photometric and visual reading for CCβ A (≥ 65 %) and CCβ B (40-65 %).

Group of anti-biotics	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	1.5	2	1.5
	Ampicillin	4	2.5	2	2.5	2.5
	Amoxicillin	4	3	2.5	3	2.5
	Cloxacillin	30	25	18	25	20
	Dicloxacillin	30	12.5	10	12.5	10
	Nafcillin	30	10	8	10	8
	Oxacillin	30	8	8	8	8
Cephalospor-ins	Cefalexin	100	*300*	*250*	*300*	*250*
	Cefapirin	60	5	5	5	5
	Cefoperazone	50	30	20	30	25
	Cefazolin	50	7	6	7	6
	Cefquinome	20	*300*	*200*	*200*	*200*
	Ceftiofur	100	*150*	100	*150*	100
	Cefalonium	20	12	10	12	10
Macrolides	Erythromycin	40	*80*	*50*	*80*	*60*
	Tylosin	50	*75*	30	*75*	40
Sulfonamides	Sulfadiazine	100	*400*	100	*300*	100
	Sulfadimethoxin	100	*600*	100	*400*	100
	Sulfamethazine	100	*>1,000*	*200*	*>1,000*	*200*
	Sulfathiazol	100	*200*	60	*200*	60
	Sulfadoxin	100	*1,500*	*300*	*400*	*300*
	Sulfamethoxypyri-dazine	100	*500*	100	*300*	100
Tetracyclines	Chlortetracycline	100	*800*	*400*	*600*	*400*
	Oxytetracycline	100	*400*	*200*	*600*	*300*
	Tetracycline	100	*600*	*300*	*400*	*300*
Aminoglyco-sides	Dihydrostreptomycin	200	*600*	*400*	*700*	*500*
	Streptomycin	200	*1,000*	*500*	*1,500*	*800*
	Gentamicin	100	*150*	80	*125*	80
	Neomycin	1,500	300	200	300	200
Fenicol	Chloramphenicol	-	*5,000*	*3,500*	*5,000*	*3,500*

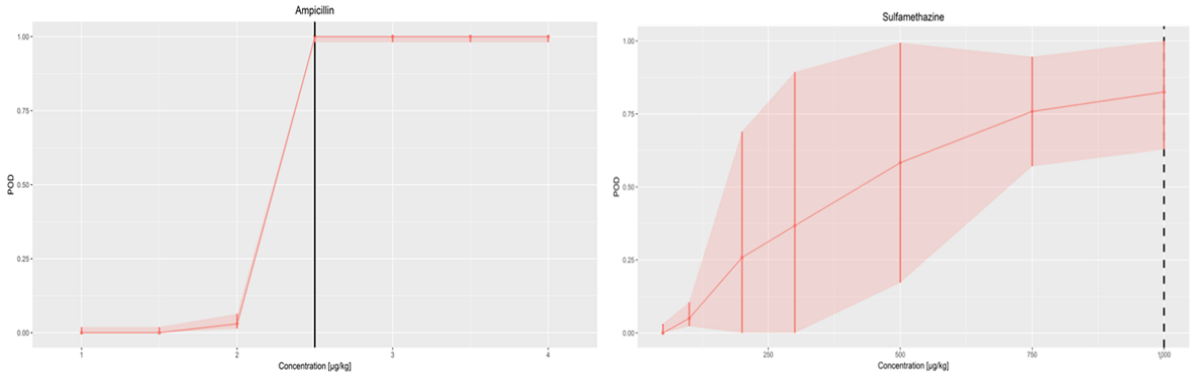
* Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

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 Ampicillin (a) and Sulfamethazine (b), combined results for photometric and visual reading.



In **Table 3**, showing results of the dose-response study for Ampicillin, it can be seen that at 2 µg/kg for photometric reading, two results equalling 3% of the total results, were clearly positive (absorbance level ≥ 65%). The additional 78 results were presumptive positive having absorbance levels of 40% - < 65 %. Adding these results to the clear positives resulted in a total of 100% CCβ B. For visual reading at 2 µg/kg, 4 results were clearly positive (Class A), but 92% of the samples were presumptive (Class B). At 2.5 µg/kg, photometric and visual reading gave 100 % clear positive (Class A) and, thus, the same (100 %) when clear positive and presumptive were combined (CCβ B).

Table 3. Dose-response for Ampicillin

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	80	0	0.00	-	-	0	0.00	80
	1.5	80	0	0.00	-	-	6	0.08	74
	2	80	2	0.03	-	-	78	1.00	0
	2.5	80	80	1.00	-	-	0	1.00	0
	3	80	80	1.00	-	-	0	1.00	0
	3.5	80	80	1.00	-	-	0	1.00	0
	4	80	80	1.00	-	-	0	1.00	0
Visual	1	120	0	0.00	-	-	0	0.00	120
	1.5	120	0	0.00	-	-	2	0.02	118
	2	120	4	0.03	-	-	106	0.92	10
	2.5	120	120	1.00	-	-	0	1.00	0
	3	120	120	1.00	-	-	0	1.00	0
	3.5	120	120	1.00	-	-	0	1.00	0
	4	120	120	1.00	-	-	0	1.00	0
Photo + visual	1	200	0	0.00	0.00	0.02	0	-	200
	1.5	200	0	0.00	0.00	0.02	8	-	192
	2	200	6	0.03	0.01	0.06	184	-	10
	2.5	200	200	1.00	0.98	1.00	0	-	0
	3	200	200	1.00	0.98	1.00	0	-	0
	3.5	200	200	1.00	0.98	1.00	0	-	0
	4	200	200	1.00	0.98	1.00	0	-	0

In **Table 4**, showing results of the dose-response study for Sulfamethazine, it can be seen that even at the highest concentration tested (1000 µg/kg), the ratio of results detected as clearly positive (absorbance level ≥ 65 %; Class A results) did not reach more than 65 % for photometric reading and 94 % for visual reading. At 200 µg/kg, for photometric reading, two results, equalling 4 % of the results, were clearly positive (absorbance level ≥ 65 %). The additional 46 results were presumptive positive having absorbance levels between 40 % - < 65 %. Adding this to the clear positives resulted in a total of 100 % CCβ B. 100% CCβ B at 200 µg/kg was also obtained for visual reading with 29 results being clear positive and 43 being presumptive.

Table 4. Dose-response for Sulfamethazine.

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	48	0	0.00	-	-	2	0.04	46
	100	48	1	0.02	-	-	27	0.58	20
	200	48	2	0.04	-	-	46	1.00	0
	300	48	5	0.10	-	-	43	1.00	0
	500	48	11	0.23	-	-	37	1.00	0
	750	48	29	0.60	-	-	19	1.00	0
	1,000	48	31	0.65	-	-	17	1.00	0
Visual	50	72	0	0.00	-	-	11	0.15	61
	100	72	5	0.07	-	-	35	0.56	32
	200	72	29	0.40	-	-	43	1.00	0
	300	72	39	0.54	-	-	33	1.00	0
	500	72	59	0.82	-	-	13	1.00	0
	750	72	62	0.86	-	-	10	1.00	0
	1,000	72	68	0.94	-	-	4	1.00	0
Photo + visual	50	120	0	0.00	0.00	0.03	13	-	107
	100	120	6	0.05	0.02	0.11	62	-	52
	200	120	31	0.26	0.00	0.69	89	-	0
	300	120	44	0.37	0.00	0.89	76	-	0
	500	120	70	0.58	0.17	0.99	50	-	0
	750	120	91	0.76	0.57	0.95	29	-	0
	1,000	120	99	0.83	0.63	1.00	21	-	0

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

In order to demonstrate that the BRT MRL Screening 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 MRL Screening 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	Confirmed positive
704	702	99.72 %	2	0.28 %	0 %	100 %

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

In this validation, 142 test plates were analysed, including 568 positive control samples and 2,840 samples of negative raw milk, in total giving 2,840 (positive control) and 14,200 (negative milk) readings, respectively, with combined photometric evaluation and visual reading.

No false-positive or false-negative results were observed when analysing the results of the negative milk samples by photometric or visual evaluation.

The maximum relative percentage value obtained with photometric reading for negative samples was 35 %, whereas the minimum relative percentage value for positive samples was 94 %. 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 MRL Screening Test was validated in an international proficiency test. 61 laboratories belonging to 55 companies originating from 10 countries participated in the examination of BRT MRL Screening 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 6**), giving a total of 945 results. No false-positive or false-negative results were observed.

Table 6. The antibiotics analysed by 61 laboratories and the percentage of correct and false results.

Antibiotic	MRL (µg/kg)	No. samples	BRT MRL Screening 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 MRL Screening Test (microtiter plate format) was tested for the 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 MRL Screening Test.