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NordVal International Certificate

Issued for:	**BRT hi-sense**
NordVal No:	053
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BRT hi-sense

Manufactured by:

Analytik in Milch Produktions- und Vertriebs-GmbH
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fulfils the validation requirements of the NordVal Validation Protocol 2. The BRT hi-sense 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 hi-sense is according to 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 hi-sense 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 3 h 30 min ± 30 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 hi-sense. 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 for Class A. However, two of the tested antiinflammatory substances, one antiparasitic and the polyether-antibiotic Monensin, were found to be presumptive positive (Class B) in 2, 3 and 6 of the 6 samples tested. Thus, when in doubt results should be confirmed.

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. 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, concentrations of veterinary drugs below these limits are accepted. According to this, the BRT hi-sense plates are applicable for the detection of the penicillin compounds: Benzylpenicillin, Ampicillin, Amoxicillin, Cloxacillin, Dicloxacillin, Nafcillin and Oxacillin, for the cephalosporins: Cefapirin, Cefoperazone, Cefazolin, Cefquinome, Ceftiofur and Cefalonium; and for Tylosin, Gentamicin and Neomycin. In addition, for photometric reading only, the method is also applicable for Sulfathiazol and Sulfamethoxypyridazine,

The method was found not to be applicable for the detection of Cefalexin, Erythromysin, Sulfadiazine, Sulfadimethoxin, Sulfamethazine, Sulfadoxin, Chlortetracycline, Tetracycline, Dihydrostreptomycin and Streptomycin 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).

Presumptive results at *CCβ B* level (40 - <65 %, **Table 1**) could be obtained for Cefalexin (photometric reading), Erythromysin, Sulfadiazine, Sulfadimethoxin and Sulfamethazine (photometric reading only), Sulfathiazol (visual reading), Sulfamethoxypyridazine (visual reading), Oxytetracycline (visual reading) and Tetracycline.

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 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	1	0.6	1	0.75
	Ampicillin	4	1.5	1.25	1.5	1.25
	Amoxicillin	4	1.5	1.25	1.5	1.25
	Cloxacillin	30	10	9	10	9
	Dicloxacillin	30	6	5	6	5
	Nafcillin	30	4	4	4	3
	Oxacillin	30	4	3	4	4
Cephalosporins	Cefalexin	100	*125*	100	*150*	*125*
	Cefapirin	60	3	2.5	3	2.5
	Cefoperazone	50	20	15	20	15
	Cefazolin	50	4	3	4	3
	Cefquinome	20	20	20	20	20
	Ceftiofur	100	10	10	15	10
	Cefalonium	20	6	5	6	5
Macrolides	Erythromycin	40	*80*	40	*80*	40
	Tylosin	50	40	20	40	25
Sulfonamides	Sulfadiazine	100	*200*	60	*200*	60
	Sulfadimethoxin	100	*200*	50	*200*	50
	Sulfamethazine	100	*400*	100	*>400*	*150*
	Sulfathiazol	100	60	40	*>100*	40
	Sulfadoxin	100	*400*	*150*	*400*	*200*
	Sulfamethoxypyridazine	100	100	40	*>200*	60
Tetracyclines	Chlortetracycline	100	*300*	*150*	*400*	*200*
	Oxytetracycline	100	100	75	*150*	75
	Tetracycline	100	*150*	75	*200*	100
Aminoglyco-sides	Dihydrostreptomycin	200	*300*	*150*	*300*	*150*
	Streptomycin	200	*500*	*250*	*500*	*300*
	Gentamicin	100	40	10	40	30
	Neomycin	1,500	150	60	150	60
Fenicol	Chloramphenicol	-	*4,000*	*2,000*	*4,000*	*2,500*

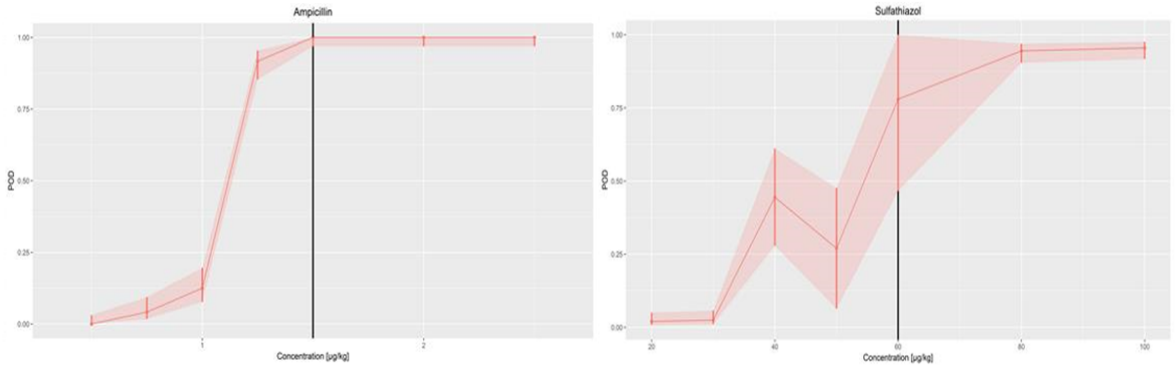
* Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

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 Sulfathiazol (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 1.25 µg/kg for photometric reading, 43 results equalling 90 % of the results were clearly positive (absorbance level ≥ 65 %). The remaining 5 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. Similarly, for visual reading at 1.25 µg/kg, 93 % of the results were clearly positive (class A), and with 5 presumptive results (Class B), this added up to 100 % of the samples being CCβ B.

At 1.5 µg/kg, both 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 res (pos. class B)	Share of CCβ B	No. of results (negative)
Photo	0.5	48	0	0.00	-	-	0	0.00	48
	0.75	48	2	0.04	-	-	6	0.17	40
	1	48	7	0.15	-	-	23	0.63	18
	1.25	48	43	0.90	-	-	5	1.00	0
	1.5	48	48	1.00	-	-	0	1.00	0
	2	48	48	1.00	-	-	0	1.00	0
	2.5	48	48	1.00	-	-	0	1.00	0
Visual	0.5	72	0	0.00	-	-	0	0.00	72
	0.75	72	3	0.04	-	-	11	0.19	58
	1	72	8	0.11	-	-	50	0.81	14
	1.25	72	67	0.93	-	-	5	1.00	0
	1.5	72	72	1.00	-	-	0	1.00	0
	2	72	72	1.00	-	-	0	1.00	0
	2.5	72	72	1.00	-	-	0	1.00	0
Photo + visual	0.5	120	0	0.00	0.00	0.03	0	-	120
	0.75	120	5	0.04	0.02	0.09	17	-	98
	1	120	15	0.13	0.08	0.20	73	-	32
	1.25	120	110	0.92	0.85	0.95	10	-	0
	1.5	120	120	1.00	0.97	1.00	0	-	0
	2	120	120	1.00	0.97	1.00	0	-	0
	2.5	120	120	1.00	0.97	1.00	0	-	0

In **Table 4**, showing results of the dose-response study for Sulfathiazol, it can be seen that even at the highest concentration tested (100 µg/kg), the ratio of results detected as clearly positive (absorbance level ≥ 65 %; Class A results) was 99 % for photometric reading and 93 % for visual reading.

The sum of the presumptive and clear positive results (positive Class A and positive Class B) adds up to 100 % CCβ B at 40 µg/kg for both photometric and visual reading.

Table 4. Dose-response for Sulfathiazol.

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 res (pos. class B)	Share of CCβ B	No. of results (negative)
Photo	20	80	3	0.04	-	-	26	0.36	51
	30	80	3	0.04	-	-	71	0.93	6
	40	80	42	0.53	-	-	38	1.00	0
	50	80	34	0.43	-	-	46	1.00	0
	60	80	76	0.95	-	-	4	1.00	0
	80	80	80	1.00	-	-	0	1.00	0
	100	80	79	0.99	-	-	1	1.00	0
Visual	20	120	1	0.01	-	-	20	0.18	99
	30	120	2	0.02	-	-	39	0.34	79
	40	120	47	0.39	-	-	73	1.00	0
	50	120	20	0.17	-	-	100	1.00	0
	60	120	80	0.67	-	-	40	1.00	0
	80	120	109	0.91	-	-	11	1.00	0
	100	120	112	0.93	-	-	8	1.00	0
Photo + visual	20	200	4	0.02	0.01	0.05	46	-	150
	30	200	5	0.03	0.01	0.06	110	-	85
	40	200	89	0.45	0.28	0.61	111	-	0
	50	200	54	0.27	0.06	0.48	146	-	0
	60	200	156	0.78	0.47	1.00	44	-	0
	80	200	189	0.95	0.90	0.97	11	-	0
	100	200	191	0.96	0.92	0.98	9	-	0

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

In order to demonstrate that the BRT hi-sense 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 hi-sense. 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, 161 test plates were analysed, including 644 positive control samples and 3,220 samples of negative raw milk, in total giving 3,220 (positive control) and 16,100 (negative milk) readings, respectively, with combined photometric evaluation and visual reading.

The analysis of negative milk samples resulted in 13 false-positive results out of 16,100 results obtained by photometric and visual evaluation, resulting in a false-positive rate of 0.1 %. No false-negative results were obtained when analysing the results of the positive samples.

Table 6. Rates of false-negative and false-positive results of the 3,220 positive controls and 16,100 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
Negative control milk	0	0	0	0	0

For photometric readings, the maximum relative percentage value obtained for negative samples was 39 %, whereas the minimum relative percentage value for positive samples was 90 %. These values demonstrate that with the chosen thresholds for photometric reading (65 % Class A; 40 % Class B, **Table 1**) the false interpretation of positive samples can be avoided, whereas false interpretation of negative samples may occur rarely.

INTERLABORATORY STUDY

The BRT hi-sense was validated in an international proficiency test. 61 laboratories belonging to 55 companies originating from 10 countries, participated in the examination of BRT hi-sense 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 5**) giving a total of 915 results.

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

Antibiotic	**MRL (µg/kg)**	**No. samples**	**BRT hi-sense**	
			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	99.8	0.2

The false-positive results (0.2 %) were reported by a single participant. No false-negative results were reported.

CONCLUSION

The BRT hi-sense (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 hi-sense.