Validation results of the AuroFlow™ PRIME Beta-Lactam MRL Assay
Cat. No: 1133-01

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1 Introduction

AuroFlow™ PRIME Beta-Lactam MRL Assay (Bioo Scientific, Austin, TX) is a competitive receptor test in dipstick format for the rapid detection of residues of β-lactams (penicillins and cefalosporins) in raw commingled cows' milk. The time to result for the one-step assay is 3 minutes.

A validation study of the test was performed at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food) in Melle, Belgium according to Commission Decision 2002/657/EC and to the guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer) (*Anonymous*, 2010).

2 Test procedure

The test was performed as indicated in the manual of the kit as given by the kit producer.
The raw milk sample is mixed to ensure sample homogeneity. The raw milk temperature should be 2-15°C. An empty tube is placed into the AuroBLOCK™ incubator (Bioo Scientific, Austin, TX). 200 µl of milk is added to the tube followed by the test strip with the arrows pointing down. After an incubation for 3 minutes at 48°C, the test strip is removed from the test tube and the results are interpreted visually by comparing the intensities of the test line and control line or by reading the strip in the QuickSTAR Strip Test Assay Reader (Bioo Scientific, Austin, TX) and applying a ratio of 1.00 as cut-off. If the T-line is less intense than the C-line, the sample is positive for β-lactams.

Table 1. Instrumental reading: interpretation of the test results.

Ratio (area measurement)	Interpretation
>1.00	"neg" (negative)
≤1.00	"pos" (positive)

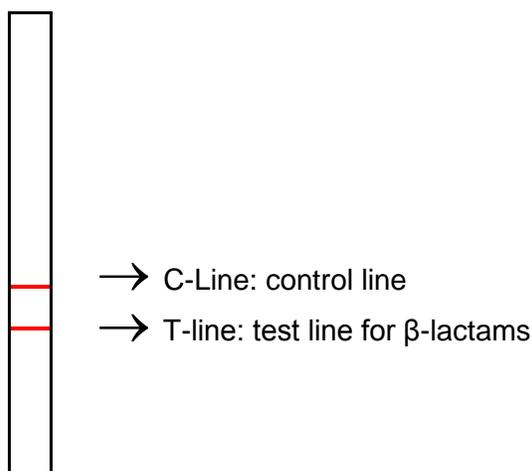→ C-Line: control line

→ T-line: test line for β-lactams

Fig. 1. Configuration of the AuroFlow™ PRIME Beta-Lactam MRL Assay dipstick.

3. Detection capability

Methods and Materials:

Spiking of antibiotic-free (blank) raw milk with β-lactam compounds
.
Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last months and which had a low to normal number of somatic cells in the milk.

- For each compound a minimum of 3 concentrations around the test sensitivity were tested.

- The increment between the concentrations tested for each compound was dependent on the level of spiking (Table 2).

- Each concentration was tested 20, 40 or 60 times in a time period of at least three days. The number of replicates is related to the closeness of the concentration tested to the respective MRL.
   - Concentration tested is <0.5 MRL: 20 times
   - Concentration tested is 0.5 – 0.9 MRL: 40 times
   - Concentration tested is 0.9 – 1.0 MRL: 60 times
   - Concentration tested is >MRL: 20 times

Table 2. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	Increment (in µg/kg)
1-10	1
10-20	2
20-50	5
50-100	10
100-250	25
250-500	50
500-1000	100
1000-5000	500

- The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

- Each day the following standards were also tested:
   blank raw milk free from antimicrobials – twice;
   blank raw milk spiked with benzylpenicillin at 1 µg/kg – twice;
   blank raw milk spiked with cefalonium at 2 µg/kg – twice.

- Detection capabilities tests were performed with Lot No 33-061 (Expiration Date March

2017), Lot No 33-063 (Expiration Date March 2017) following the manufacturer's instructions. The intensity of color formation of the test line was compared to the intensity of the control line and was interpreted by means of a QuickSTAR Strip Test Assay Reader. All results (reader values) were collected in a data base.

Results:

A summary of the detection capabilities is given in Table 3.

Table 3. Detection capability (µg/kg) of AuroFlow™ PRIME Beta-Lactam MRL Assay for β-lactams in raw bovine milk. Interpretation of strips with QuickSTAR Strip Test Assay Reader. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Antibiotic Group/ antibiotic	Marker residue	EU MRL (µg/kg)	Detection capability (µg/kg)
penicillins			
benzylpenicillin	benzylpenicillin	4	2
ampicillin	ampicillin	4	4
amoxicillin	amoxicillin	4	4
oxacillin	oxacillin	30	3
cloxacillin	cloxacillin	30	3
dicloxacillin	dicloxacillin	30	3
nafcillin	nafcillin	30	7
cefalosporins			
ceftiofur	ceftiofur & desfuroylceftiofur	100[a]	90 **700**
cefquinome	cefquinome	20	8
cefazolin	cefazolin	50	**60**
cephapirin	cephapirin & desacetylcephapirin	60[c]	7 25
cefacetrile	cefacetrile	125	25
cefoperazone	cefoperazone	50	3
cefalexin	cefalexin	100	**>1,000**
cefalonium	cefalonium	20	2
cefuroxime		---[c]	**350**

Notes: indicated in red and bold: ccβ > MRL.
EU MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 01/09/2016).
[a]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur,

b: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin,
c: no MRL fixed for cefuroxime in milk.

Discussion:

The marker residues of all β-lactams with a MRL in milk (EU-Regulation 37/2010 and amendments) can be detected by the AuroFlow™ PRIME Beta-Lactam MRL Assay at their respective MRL except for three marker residues: desfuroylceftiofur (CCβ=700 µg/kg, MRL=100 µg/kg); cefazolin (CCβ=60 µg/kg, MRL=50 µg/kg)), and cefalexin (CCβ=>1,000 µg/kg, MRL=100 µg/kg).
The detection capability for penethamate in milk was not tested since penethamate is not stable in milk and is quickly converted to benzylpenicillin; hence benzylpenicillin is the marker residue.

4. Test selectivity

Methods and Materials:

The selectivity of AuroFlow™ PRIME Beta-Lactam MRL Assay was tested by analyzing milk doped with compounds belonging to families of antibiotics or chemotherapeutics other than β-lactams. Raw milk was doped at a high concentration (100x MRL or 100x MRPL in milk) in raw milk. All testing was completed in duplicate.

A summary of the test selectivity data is given in Table 4.

Table 4. Selectivity of the AuroFlow™ PRIME Beta-Lactam MRL Assay, QuickSTAR Strip Test Assay Reader results.

Compound	MRL (µg/kg)	AuroFlow™ PRIME Beta-Lactam MRL Assay	
		Conc. spiked in milk (µg/kg)	Ratio (avg)
oxytetracycline	100	10,000	9.89
erythromycin	40	4,000	8.99
enrofloxacin	100[a]	10,000	4.99
chloramphenicol	--[b]	30	13.04
neomycin B	1,500	150,000	11.56
colistin	50	5,000	7.67
lincomycin	150	15,000	14.35
clavulanic acid	200	20,000	0.00
sulfadiazine	100	10,000	7.02
trimethoprim	50	5,000	9.71
dapsone	--[c]	500	8.88

Notes: avg: average; [a] sum of enrofloxacin and ciprofloxacin should not exceed the MRL of 100 µg/kg; [b]

Minimum Required Performance Limit for testing chloramphenicol in milk is 0.3 µg/kg; [c] recommended concentration for testing dapsone in milk is 5 µg/kg.

Discussion:
When challenged with high concentrations of non-target drugs, the AuroFlow™ PRIME Beta-Lactam MRL Assay was found to be highly specific for its target drug class. No interference by compounds not belonging to the group of β-lactams except for clavulanic acid. The interaction of clavulanic acid, a β-lactamase inhibitor, is expected since this molecule contains a β-lactam structure resembling that of penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anonymous*, 2005). The detection capability (min 95 % positive results) of clavulanic acid in milk = 900 µg/kg (MRL=200 µg/kg).

AuroFlow™ PRIME Beta-Lactam MRL Assay is a highly specific test for detection of β-lactams in milk and does not detect compounds from the tetracyclines, macrolides, quinolones, amphenicols, aminoglycosides, polymyxins, lincosamides, sulfonamides and diamino pyrimidine derivatives families, nor dapsone.

5. Test robustness

5.1. Repeatability of the reader

Methods and Materials:

Blank and positive milk samples (2 levels) were analyzed and the strips were then dried to ensure completeness of color formation. The dried strips were then measured 10 times with the QuickSTAR Strip Test Assay Reader. The standard deviation of repeatability and coefficient of variation of the reader was calculated.

Results:

Results of the repeatability trial for the QuickSTAR Strip Test Assay Reader are summarized in Table 5.

Table 5. Repeatability of the QuickSTAR Strip Test Assay Reader at different ratio levels (based on 10 replicates per case).

	n	mean ratio	S_r	CV(%)
blank	10	7.93	0.14	1.72
low positive	10	0.80	0.05	6.18
high positive	10	0.28	0.02	6.34

Notes: s_r, standard deviation of repeatability; CV, coefficient of variation
Discussion:

The reader variation is as expected with a variance of about 5% which are acceptable values. A somewhat higher variation for doped milk results are fully acceptable. It is worth noting that even for the low positive results with ratio values close to the cut-off value no negative results were observed.

5.2. Repeatability of the test

Methods and Materials:

Blank and positive milk samples (2 levels) were analyzed (10 replicates each). The repeatability of the test was calculated. All dipsticks were read directly after incubation. Standard deviation of repeatability and coefficient of variation of the test were calculated.

Results:

The results of the repeatability of the test experiment are summarized in Table 6.

Table 6. Repeatability of the AuroFlow™ PRIME Beta-Lactam MRL Assay at different ratio levels with reading of the strips by means of an QuickSTAR Strip Test Assay Reader (based on 10 replicates per case).

	n	mean ratio	S_r	CV(%)
blank	10	9.02	2.23	24.77
low positive BL	10	0.80	0.10	12.44
high positive BL	10	0.25	0.04	14.04

Notes: s_r, standard deviation of repeatability; CV, coefficient of variation

Discussion:

For test variation a variance of about 20% could be expected and accepted. The AuroFlow™ PRIME Beta-Lactam MRL Assay is giving higher values for negative results, however no false positive results were obtained since the ratio values (mean ratio = 9.02) are far away from the cut-off ratio of 1.00. For positive results variances <20% were encountered and are very acceptable.
The purpose of evaluating the high and low positive levels was strictly for evaluating repeatability at different reader responses and does not imply the reader is able to quantitate levels of contamination. Without the knowledge of the contaminating compound it is not possible to correlate reading intensity with concentrations.

5.3. Length of incubation

Methods and Materials:

In order to determine the robustness of the assay, the length of incubation was perturbed. According to the manufacturer's instructions, the assays should be incubated for 3 minutes. In this test for assay robustness, three blank milk samples and three positive milk samples (milk doped with benzylpenicillin at 2 µg/kg or cefalonium at 2 µg/kg, respectively) were analyzed with deviations of +/- 15 seconds of the standard 3 minutes incubation time.

Results:

The results of the influence of the length of incubation on the AuroFlow™ PRIME Beta-Lactam MRL Assay are summarized in Table 7.

Table 7. Ratios for AuroFlow™ PRIME Beta-Lactam MRL Assay obtained with the QuickSTAR Strip Test Assay Reader when testing blank and doped milk samples after application of different incubation times.

	Length of incubation		
	3 min = ref	**2min 45sec**	**3min 15sec**
AuroFlow™ PRIME Beta-Lactam MRL Assay ratio's			
blank milk			
mean	6.43	5.97	4.75
min	5.25	4.22	4.06
max	7.44	7.68	5.42
milk + 2 µg/kg benzylpenicillin			
mean	0.09	0.08	0.04
min	0.07	0.05	0.03
max	0.12	0.11	0.07
milk + 2 µg/kg cefalonium			
mean	0.24	0.45	0.31
min	0.17	0.27	0.15
max	0.27	0.78	0.43

Notes: min: minimum, max: maximum.

Discussion:

Performing the AuroFlow™ PRIME Beta-Lactam MRL Assay with incubation times different from the standard 3 minutes had no impact on test results for blank and doped milk; all results remain clearly negative or positive, respectively. The detection is not hampered or influenced by a ±9% too short or too long incubation.

5.4. Influence of waiting time on reader results

Methods and Materials:

Blank and doped milk samples (benzylpenicillin at 2 µg/kg or cefalonium at 2 µg/kg) were analyzed with the AuroFlow™ PRIME Beta-Lactam MRL Assay and the strips were read with the QuickSTAR Strip Test Assay Reader immediately after incubation and after 1 minute, 5 minutes or 15 minutes after the incubation time was complete. Three samples of each type were tested under each condition.

Results:

The results of the influence of the waiting time on test results are summarized in Table 8.

Table 8: Ratios obtained when testing blank and doped milk samples and reading the strips directly after incubation or with a delay of 1, 5 and 15 minutes respectively. Results obtained with the QuickSTAR Strip Test Assay Reader.

	Delay of reading			
	direct = ref	**1 min**	**5 min**	**15 min**
AuroFlow™ PRIME Beta-Lactam MRL Assay ratio's				
blank milk				
mean	6.00	5.95	5.88	6.07
min	5.28	5.14	5.24	5.76
max	6.38	6.39	6.27	6.52
milk + 2 µg/kg benzylpenicillin				
mean	0.07	0.06	0.05	0.03
min	0.04	0.03	0.03	0.02
max	0.10	0.08	0.06	0.04
milk + 2 µg/kg cefalonium				
mean	0.40	0.32	0.24	0.15
min	0.26	0.21	0.17	0.11
max	0.63	0.50	0.00	0.22

Notes: min: minimum, max: maximum.

Discussion:

A delay of reading even up to 15 minutes has no significant effect on the AuroFlow™ PRIME Beta-Lactam MRL Assay results. All blank milk and doped milk samples produced expected negative or positive results. For the doped samples we even remark a tendency to lower ratios (increased detection capability).

5.5. Influences in variation of volume of milk

Methods and Materials:

Blank milk and milk doped with benzylpenicillin at 2 µg/kg or cefalonium at 2 µg/kg were analyzed to measure the effects of +/- 10% differences in milk sample volume on the AuroFlow™ PRIME Beta-Lactam MRL Assay. Three samples of each type were tested under each condition.

Results:

The results of the influence of milk sample volume on test results are summarized in Table 9.

Table 9: Ratios obtained when testing different volumes (180, 200 or 220 µl, respectively) of milk. Results obtained with the QuickSTAR Strip Test Assay Reader.

	Volume of milk		
	200 µl = ref	180 µl	220 µl
AuroFlow™ PRIME Beta-Lactam MRL Assay ratio's			
blank milk			
mean	5.42	4.16	5.86
min	4.14	3.32	4.16
max	6.23	4.62	7.59
milk + 2 µg/kg benzylpenicillin			
mean	0.16	0.11	0.14
min	0.11	0.05	0.11
max	0.20	0.19	0.19
milk + 2 µg/kg cefalonium			
mean	0.94	0.64	0.29
min	0.50	0.55	0.21
max	1.40	0.76	0.35

Notes: min: minimum, max: maximum.

Discussion:

Varying milk sample volume by +/- 10% (20 µL) did not produce a noticeable effect on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. For the milk doped with cefalonium at 2 µg/kg lower ratio values were obtained when the milk volume applied in the test was 10% deviating from the normal volume of 200 µl.

5.6. Influences in variation of milk temperature

Methods and Materials:

Blank milk and milk doped with benzylpenicillin at 2 µg/kg or cefalonium at 2 µg/kg were

analyzed to measure the effects of different milk temperature at the time of testing (4°C reference, 10°C, 15°C, and 20°C). Three samples of each type were tested under each condition.

Results:

Results of the influence of milk sample temperature on the AuroFlow™ PRIME Beta-Lactam MRL Assay test are summarized in Table 10.

Table 10: AuroFlow™ PRIME Beta-Lactam MRL Assay ratios obtained for blank and doped milk with the QuickSTAR Strip Test Assay Reader when testing different milk temperatures (10, 15 and 20°C versus 2-4°C).

	Temperature of milk			
	2-4°C = ref	10°C	15°C	20°C
AuroFlow™ PRIME Beta-Lactam MRL Assay ratio's				
blank milk				
mean	6.33	3.82	4.56	3.67
min	3.12	3.75	3.39	2.95
max	8.23	3.87	6.50	4.17
milk + 2 µg/kg benzylpenicillin				
mean	0.07	0.04	0.05	0.11
min	0.05	0.03	0.04	0.04
max	0.09	0.06	0.07	0.22
milk + 2 µg/kg cefalonium				
mean	0.45	0.35	0.40	0.41
min	0.32	0.24	0.27	0.24
max	0.54	0.54	0.62	0.74

Notes: min: minimum, max: maximum.

Discussion:

In general, variation in milk temperature did not affect the performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. For blank milk samples all tests produced negative results despite lower ratio values were obtained when testing milk with a temperature above 2-4°C. Variations in milk temperature did not appear to produce changes in ratios for tests of doped, positive milk.

5.7. Influences of milk quality and composition

Methods and Materials:

The effect of milk quality and composition on performance of the AuroFlow™ PRIME Beta-

Lactam MRL Assay was measured. Compared to normal raw milk, variables included high somatic cell count, high bacterial count, low and high fat content, low and high protein content, and low and high pH. Samples included blank milk and milk doped with benzylpenicillin at 2 μg/kg or cefalonium at 2 μg/kg. Ten samples of each type were tested under each condition.

Results:ty

Results are presented as a series of charts in Figures 3a to 3c. The legend list below describes the sample type tested and is intended for each of the following milk quality and composition graphs.

Legend:1= reference: normal raw milk;
2= Somatic cell count >10^6/ml;
3= High bacterial count (>$5×10^5$/ml);
4= Low protein (<3 g/100 ml);
5= High protein (>4 g/100 ml);
6= Low pH (6.0);
7= High pH (7.5).

- ● mean ratio
- ▼ minimum ratio
- ■ maximum ratio
- ── cut-off (ratio 1,0)
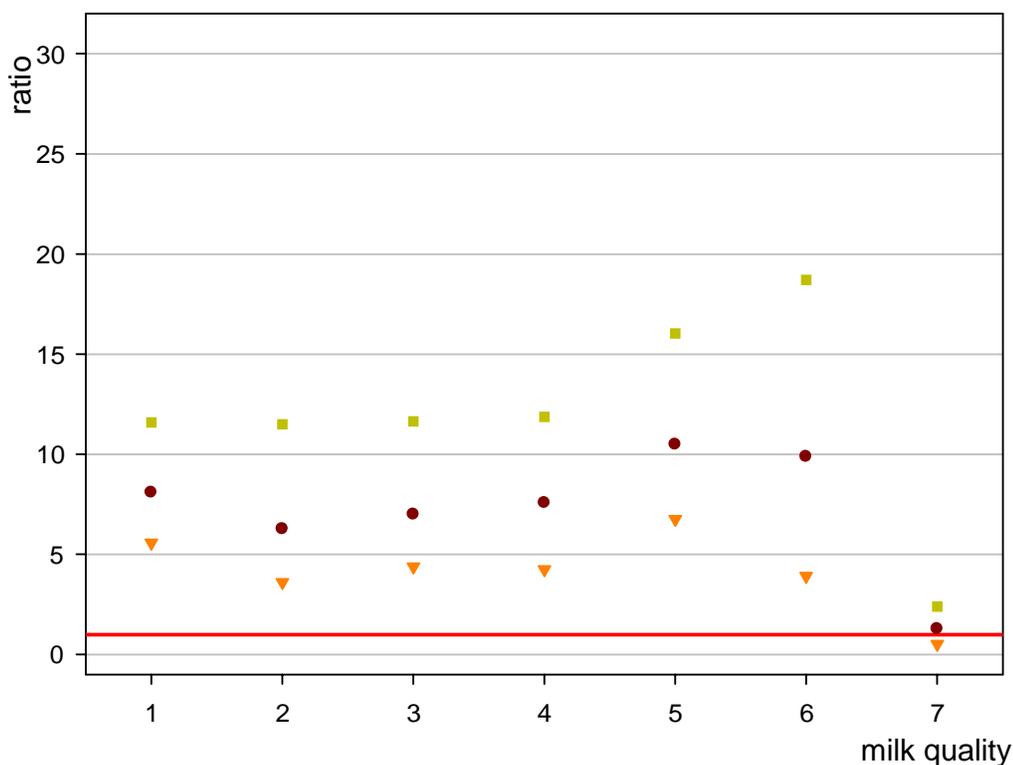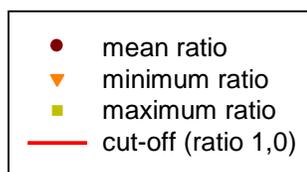
Figure 3a. Effect of the quality and composition of blank milk on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. QuickSTAR Strip Test Assay Reader results (ratio's).
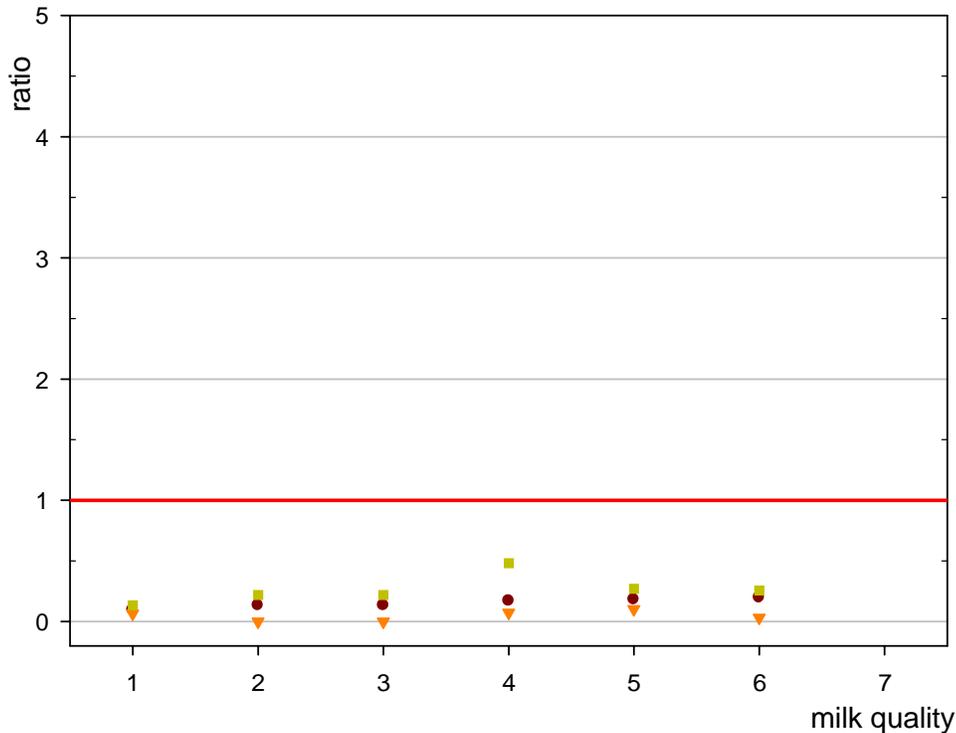
Figure 3b. Effect of the quality and composition of milk doped with 2 µg/kg of benzylpenicillin on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. QuickSTAR Strip Test Assay Reader results (ratio's).
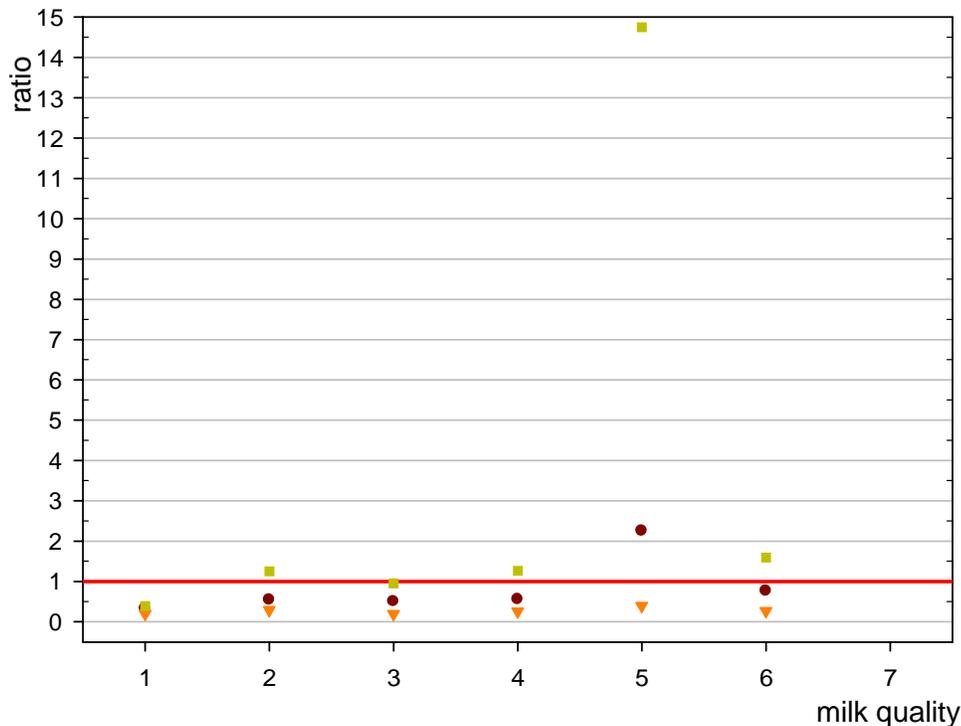

Figure 3c. Effect of the quality and composition of milk doped with 2 µg/kg of cefalonium on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. QuickSTAR Strip Test Assay Reader results (ratio's).

Discussion:

Testing of blank milk of an abnormal quality/composition resulted in correct results except for milk with an extreme high pH of 7.5. Note that milk with such a high pH could only be individual cows' milk from an animal with chronic mastitis. Farm tank milk or tanker milk with a pH = 7.5 is not existing in practice.

Since even false positive results occurred for milk with a pH of 7.5, such milk was not doped. All milk samples with an abnormal quality/composition and doped with benzylpenicillin at 2 μg/kg gave correct positive results. For the milk samples with abnormal quality/composition and doped with cefalonium at 2 μg/kg in general slightly higher ratio values were obtained compared to the values measured for the reference milk samples. One of the samples with a high protein content and doped with cefalonium gave a very high ratio of 14.72 (retested 14.32); two other samples also tested (borderline) negative (ratio's 1.66 and 1.26) The high negative ratio is possibly caused by a too slow and difficult flow over the dipstick resulting in a very weak reference line.
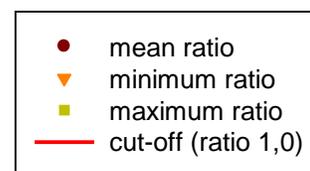

5.8. Type of milk

Methods and Materials:

The effect of type of milk on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay test was measured. In addition to normal raw bovine milk (reference condition), the following types of milk were tested: UHT, sterilized, reconstituted powder, and frozen-thawed milk. Samples included blank milk and milk doped with benzylpenicillin at 2 μg/kg or cefalonium at 2 μg/kg..

Results:

Results are presented as a series of charts in Figures 4a to 4c. The list below describes the sample type tested and is intended for each of the following graphs.

Legends: 1= reference: normal raw milk;
2= UHT;
3= sterilized;
4= reconstituted milk powder;
5= frozen-thawed;

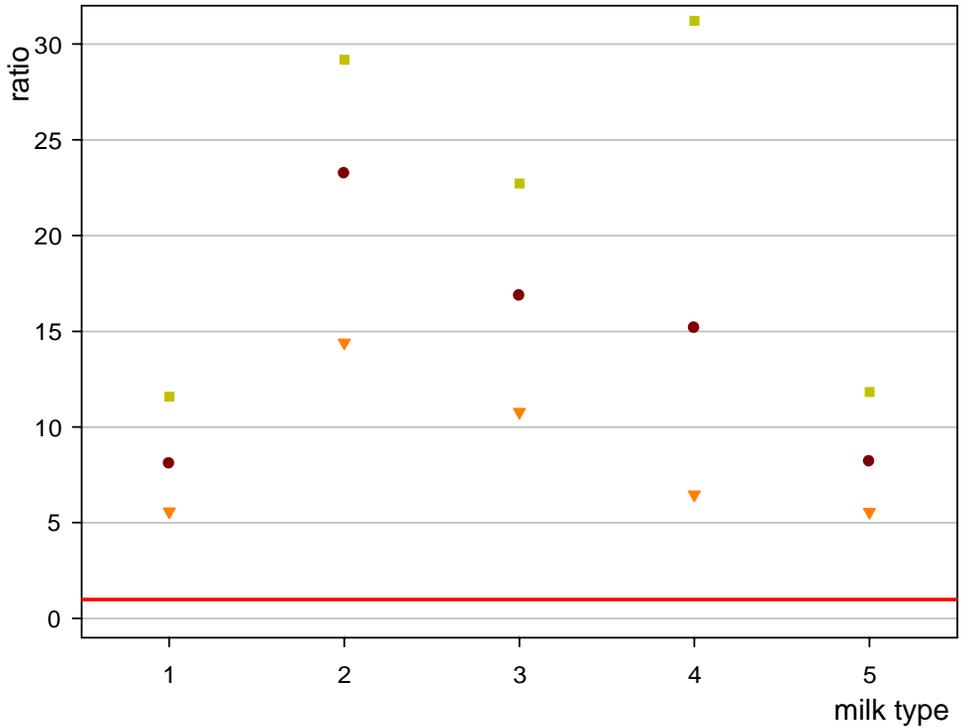|	
●	mean ratio
▼	minimum ratio
■	maximum ratio
——	cut-off (ratio 1,0)

Figure 4a. Effect of type of blank milk on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. QuickSTAR Strip Test Assay Reader results (ratio's).
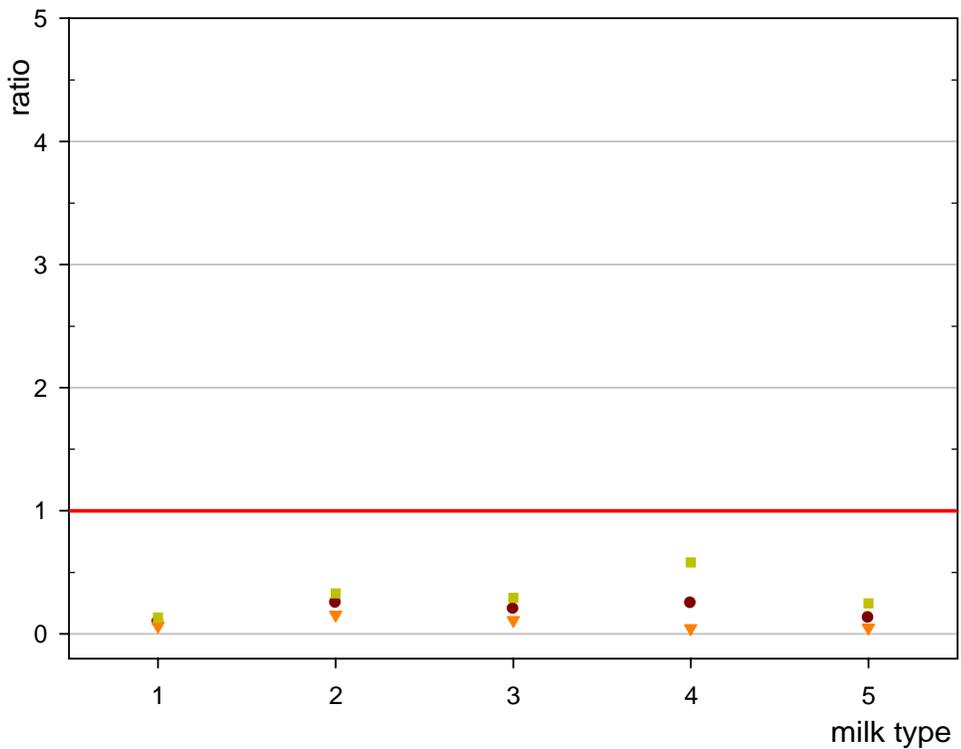

Figure 4b. Effect of type of milk doped with 2 µg/kg of benzylpenicillin on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. QuickSTAR Strip Test Assay Reader results (ratio's).
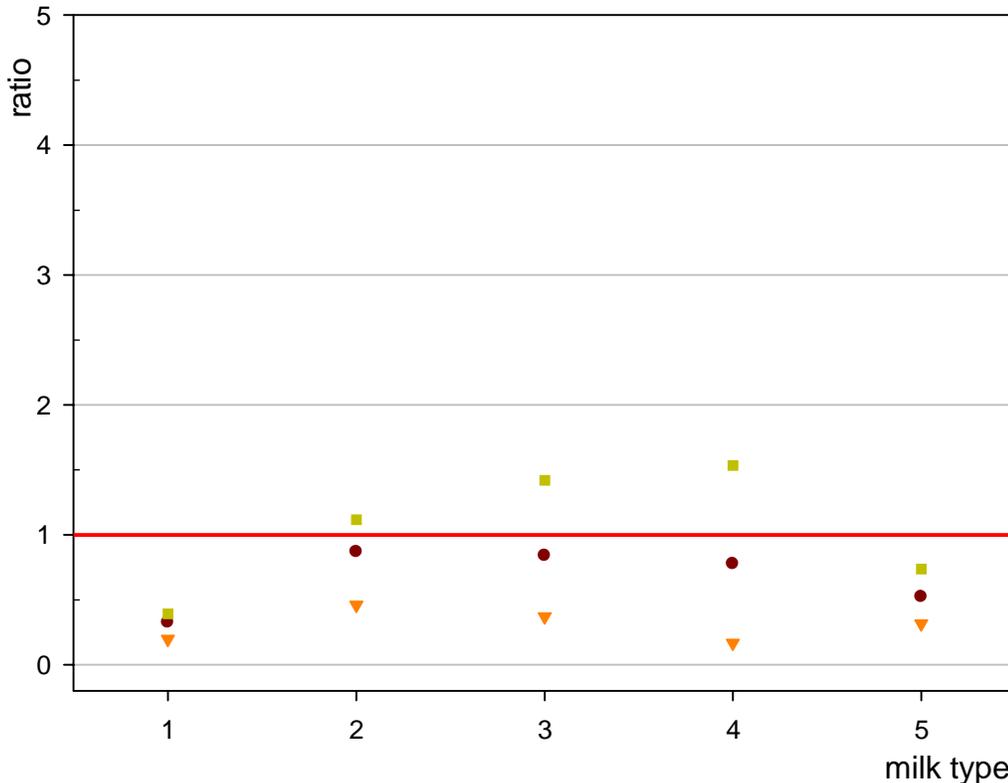
Figure 4c. Effect of type of milk doped with 2 µg/kg of cefalonium on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. QuickSTAR Strip Test Assay Reader results (ratio's).

Discussion:

Testing of blank heat-treated milk resulted in a bigger variation in ratio values compared with the reference values obtained for raw cows' milk. In general for all tested types of milk correct negative results were obtained, even more, higher (more negative) ratio values were observed for the heat treated milk samples.

In the doped milk samples we remark no problems for the detection of 2 µg/kg of benzylpenicillin in the different milk types. The detection of cefalonium is slightly hampered in heat treated milk or reconstituted milk powder since higher ratio values were obtained but all below 1.53. Notice that 2 µg/kg of cefalonium was tested, a concentration far below MRL; so detection at MRL (20 µg/kg) is certainly no problem. Besides the small shift in detection capability for cefalonium some problems with the reading were noticed when testing UHT milk. Six samples caused an 'invalid reading'; for 5 samples a second reading gave a correct reading result.

5.9. Test for false-positive results

Methods and Materials:

Three hundred farm milk samples and 300 comingled tanker milk samples were analyzed by AuroFlow™ PRIME Beta-Lactam MRL Assay to determine the false positive rate of the test in

testing of routine raw milk samples. Samples giving a positive screening result were further analysed with other tests or LC-MS/MS.

Results and discussion:
All 300 blank farm milk samples tested negative on the AuroFlow™ PRIME Beta-Lactam MRL Assay. One tanker milk sample (RMO248) was giving a positive result (ratio=0.34) due to a bad line formation on the strip. Hence the total rate of false positive results = 1 milk sample upon 600 milk samples = 0.17%, showing the AuroFlow™ PRIME Beta-Lactam MRL Assay to be highly specific in routine testing of raw milk samples.

It is worth noting that the incurred tanker milk sample RMO137 containing 0.8 µg/kg of benzylpenicillin (LC-MS/MS result) tested borderline negative (ratio=1.15) on the AuroFlow™ PRIME Beta-Lactam MRL Assay. This milk tested positive on some other tests, e.g. on the beta-lactam channel of the BetaStar® 4D, but due to a concentration below the CCβ (Table 3) the negative result is not considered as a false negative result.

5.10. Lot-to-Lot Variability

Methods and Materials:

The following samples were analyzed at the same time with 2 different batches of AuroFlow™ PRIME Beta-Lactam MRL Assay reagents, namely lot 33-0361 and lot 33-063.
- Blank milk (antibiotic-free raw milk) (20 different samples)
- Positive raw milk doped with benzylpenicillin at 2 µg/kg (20 different samples)
- Positive raw milk doped with cefalonium at 2 µg/kg (20 different samples).
The results are presented in Table 11.

Table 11. Ratios obtained for blank and positive milk with two different batches of AuroFlow™ PRIME Beta-Lactam MRL Assay reagents (lot 33-061 and 33-063) and QuickSTAR Strip Test Assay Reader (based on 20 replicates per case).

Batch differences				
Lot	sample	Ratio values		
		mean	min	max
	blank	5.96	1.44	8.77
33-061	benzylpenicillin 2 µg/kg	0.12	0.04	0.24
	cefalonium 2 µg/kg	0.31	0.05	0.56
	blank	6.79	1.45	11.59
33-063	benzylpenicillin 2 µg/kg	0.09	0.02	0.17
	cefalonium 2 µg/kg	0.31	0.09	0.44

Notes: min: lowest ratio; max: highest ratio.

Discussion:

No significant differences were noted between both batches of reagents for negative samples and for spiked samples.

6. Interlaboratory testing - National ring trial

Methods and Materials:

Twice a year, ILVO-T&V organizes a national ring trial for the Belgian dairy industry regarding the detection of antibiotic residues in milk by microbiological and rapid tests. In the ring trial of Spring and Autumn 2016 the AuroFlow™ PRIME Beta-Lactam MRL Assay was integrated as a rapid test. The drugs and concentrations of blind-coded doped raw milk samples distributed in the ring trial of April 21, 2016 and October 27, 2016 are shown in Table 12 and 14, respectively. Results were determined using a QuickSTAR Strip Test Assay Reader.

Results:

Results obtained with the AuroFlow™ PRIME Beta-Lactam MRL Assay of the ring trial of April 21, 2016 and October 27, 2016 are presented in Table 13 and 15, respectively.

Table 12. Sample identification for the blind-coded raw milk samples of the ring trial organized by ILVO-T&V on April 21, 2016 (Ooghe and Reybroeck, 2016a).

Sample	Drug	Concentration (µg/kg)	MRL (µg/kg)
I	benzylpenicillin/ thiamphenicol	4 / 50	4 / 50
J	Ceftiofur	100	100
K	Doxycycline	40	---[a]
L	Amoxicillin	4	4
M	blank	---	---
N	benzylpenicillin	2	4
O	cloxacillin	30	30
P	Benzylpenicillin / chloramphenicol	3 / 0.3	4 / 0.3

Note: [a]: not allowed to be given to cows producing milk for human consumption.

Table 13. Results for AuroFlow™ PRIME Beta-Lactam MRL Assay in the ring trial organized by ILVO-T&V on April 21, 2016 (Ooghe and Reybroeck, 2016a).

Sample	Visual reading	Instrumental reading		Final result
		Ratio	Result	
I	+	0.01	+	+
J	+	0.62	+	+
K	-	7.88	-	-
L	+	0.73	+	+
M	-	9.05	-	-
N	+	0.14	+	+
O	+	0.00	+	+
P	+	0.02	+	+

Notes: - negative; +: positive. Kit lot number 33-061 (expiration March 2017). QuickSTAR Strip Test

Assay Reader.

Table 14. Sample identification for the blind-coded raw milk samples of the ring trial organized by ILVO on October 27, 2016 (Ooghe and Reybroeck, 2016b).

Sample	Drug	Concentration (µg/kg)	MRL (µg/kg)
I	oxytetracycline/dihydrostreptomycin	100 / 200	100[a] / 200
J	cloxacillin	30	30
K	cefazolin	50	100
L	cefalexin	100	100
M	benzylpenicillin	1	4
N	benzylpenicillin / florfenicol	3 / 10	4 / ---
O	nafcillin	30	30
P	benzylpenicillin	4	4

Notes: MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 01/01/2016); [a]: sum of chlortetracycline and the 4-epimer of chlortetracycline.

Table 15. Results for AuroFlow™ PRIME Beta-Lactam MRL Assay in the ring trial organized by ILVO-T&V on October 27, 2016 (Ooghe and Reybroeck, 2016b).

Sample	Visual reading	Instrumental reading		Final result
		Ratio	Result	
I	-	7.13	-	-
J	+	0.00	+	+
K	+	0.66	+	+
L	-	4.48	-	-
M	-	1.12	-	-
N	+	0.01	+	+
O	+	0.02	+	+
P	+	0.01	+	+

Notes: - negative; +: positive. Kit lot number 33-063 (expiration March 2017). QuickSTAR Strip Test Assay Reader.

Discussion:

In both ring trials no false positive results were obtained. β-lactam antibiotics were detected correctly in samples I, J, L, N, O and P (ring trial of April 2016) and in samples J, K, N, O and P (ring trial of October 2016). β-lactam sample test results were consistent with test capability with proper reader interpretations. The method performed in the national ring trial as it was validated: benzylpenicillin at 1 µg/kg in milk (sample M, October 2016) is not detected (ccβ = 2 µg/kg) but is giving a lower ratio (1.12) compared to blank milk (ratio =7.13).

7. Daily control samples

Methods and Materials:

The following control samples were analyzed daily with the AuroFlow™ PRIME Beta-Lactam MRL Assay and QuickSTAR Strip Test Assay Reader:
  blank raw milk free from antimicrobials
  blank raw milk spiked with benzylpenicillin at 1 µg/kg
  blank raw milk spiked with cefalonium at 2 µg/kg

Results:

The results obtained for the daily control samples are presented in Figures 5a to 5c.

Discussion:

Results indicate no false positive results for blank control samples. Benzylpenicillin doped in milk at 1 µg/kg (< CCβ) is causing some negative results but all ratio values were below 2.5. Two samples out of 73 samples of milk containing 2 µg/kg of cefalonium gave a negative borderline result (twice a ratio of 1.45). Daily fluctuations are noticed both for blank and doped samples.
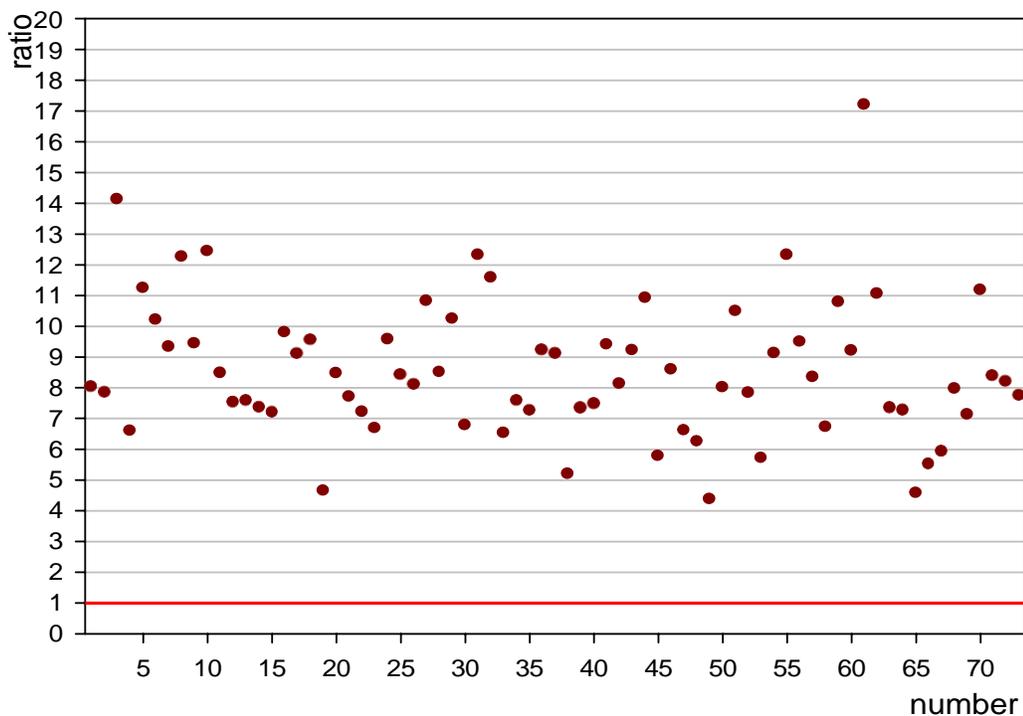
Figure 5a. QuickSTAR Strip Test Assay Reader results of daily control samples: blank milk.
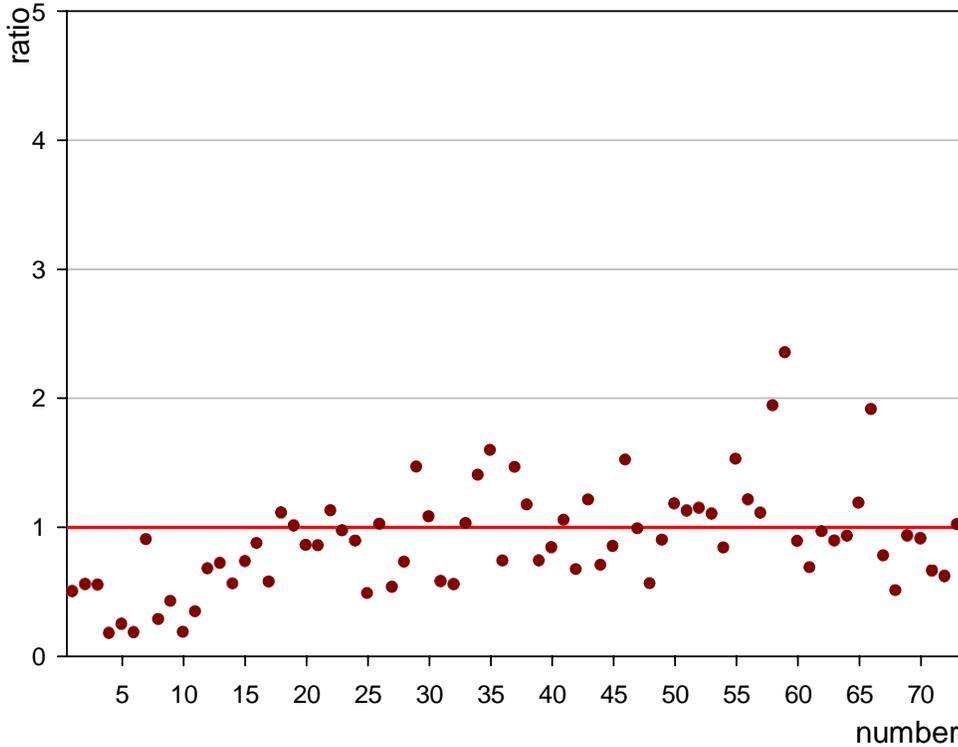
Figure 5b. QuickSTAR Strip Test Assay Reader results of daily control samples: milk doped with 1 µg/kg of benzylpenicillin.
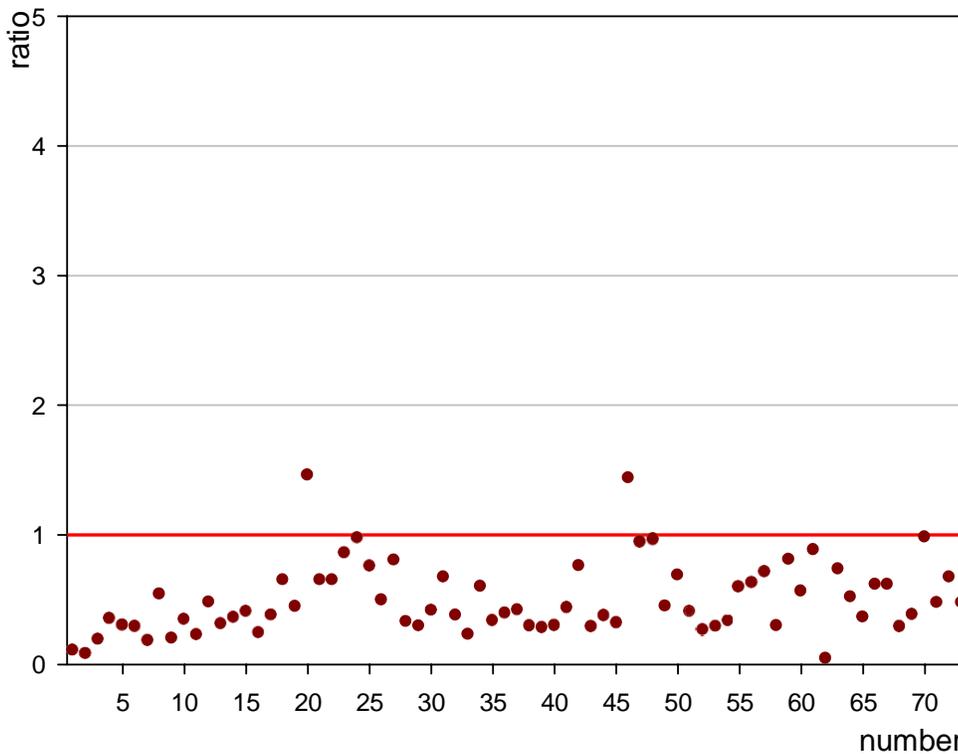

Figure 5c. QuickSTAR Strip Test Assay Reader results of daily control samples: milk doped with 2 µg/kg of cefalonium.

8. Other remarks and observations

When the flow of the milk over the dipstick is poor (high fat, high protein,…) an abnormal control line could be obtained.

Once we observed an abnormal reader result due to a bad positioning of the strip. However, no warning was given by the reader. It is advisable that bad positioning of strips is always detected by the reader and reported as an invalid result.

9. End conclusions

The AuroFlow™ PRIME Beta-Lactam MRL Assay is a lateral flow test for β-lactam residues in commingled raw cows' milk giving a result in 3 minutes. The AuroFlow™ PRIME Beta-Lactam MRL Assay could detect the marker residues of all β-lactams with a MRL in milk (EU-Regulation 37/2010 and amendments) at their respective MRL in minimal 95% of the cases except for desfuroylceftiofur, cefazolin, and cefalexin. The test is very specific for the class of β-lactam antibiotics; only an interference by clavulanic acid was noticed. A good reader and test repeatability were obtained. Small deviations in incubation time had no impact on the test results both for negative and positive samples. No significant impact by a delay of the reading (up to 15 minutes) was noticed on the test results of blank and doped milk samples. Varying milk sample volume by +/- 10% (20 µL) did not produce a noticeable effect on performance of the AuroFlow™ PRIME Beta-Lactam MRL Assay. In general, variation in milk temperature did not affect the performance of the test.

The kit was efficient in different normal raw cows' milks and in specific extreme milks specially selected (or prepared) for this study (somatic cells, total bacterial count, protein, pH) except for milks with an extreme high pH of 7.5 (false positive results). The test also works for heat-treated milk or reconstituted milk powder. Some difficulties were encountered testing UHT milk by obtaining invalid results. In addition the kit demonstrated an overall good performance (0.17% of false positive results) on a wide panel of 300 individual farm and 300 tanker milk samples as well as during two national ring test trials. In case of a positive result, it is recommended to check the lines on the strip if they are well formed. Some day to day variations were noticed for both blank and doped milk. No differences in detection capability were found between two different batches of reagents. Very good results were obtained with the test in two national ring trials.

The validation results indicate that the AuroFlow™ PRIME Beta-Lactam MRL Assay is appropriate for use as a screening test for β-lactams in raw and heat treated commingled cows' milk.

REFERENCES

Anonymous. 2010. Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer). Community Reference Laboratories Residues (CRLs). 20/01/2010: 1-18.

AuroFlow™ PRIME Beta-Lactam MRL Assay kit insert, Bioo Scientific, Austin, TX.

Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Comm. 2002 L221: 8-36.

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. Off. J. Eur. Union 2010 L15: 1-72.

Ooghe S., Reybroeck W. 2016a. Rapport ringonderzoek antibioticascreening in melk: sneltesten en microbiologische testen. 21 april 2016. T&V-ILVO, Melle, Belgium: 1-30.

Ooghe S., Reybroeck W. 2016b. Rapport ringonderzoek antibioticascreening in melk: sneltesten en microbiologische testen. 27 oktober 2016. T&V-ILVO, Melle, Belgium: 1-36.

Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Union 2009 L152: 11-22.