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***Validation report of
Milksensor® BT10i
(β -lactams & Tetracyclines)
(Product N° BT10i)***

(Mayasan A.S., Istanbul, Turkey)

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1. Introduction

Validation report of Milksensor® BT10i (β -lactams & Tetracyclines) Test Kit (Mayasan A.S., Istanbul, Turkey). The test is a colloidal gold immunochromatography two-step 8-min (3'+5') rapid lateral flow assay to detect β -lactam and tetracyclines antibiotic residues in raw commingled cows' milk.

This new test was validated at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food) according to Commission Implementing Regulation 2021/808 and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). The following analytical parameters were checked: detection capability, test specificity and test robustness (impact of deviation of the test protocol, impact of the milk composition or milk type). The test will also be included in the next interlaboratory study organised by ILVO in spring 2022.

2. Test procedure

Test preparation

Bring the test kit and samples to room temperature with tubes closed to avoid moisture and sunlight. Prepare the milk sample at room temperature which is fresh and free from precipitation and clotting.

The test can also be used for the testing of milk powder. Then the sample should be prepared by diluting 1:9 (10 g of milk powder and 90 ml of water). This was however not tested in this validation, only raw milk was used.

Test procedure

1st step: Turn on the metal incubator and wait until it is stable at 42°C.

2nd step: Add 200 μ l milk to a microwell and mix well by pipetting up and down 10 times.

3rd step: Place the micro-wells in the incubator, and incubate for 3 minutes at 42°C. During this time period place the dipsticks ready onto the incubator.

4th step: At the end of the first incubation, the dipsticks will fall down and automatically the second incubation will start (5 minutes at 42°C).

5th step: Take out the strip from the well and remove the sample pad at the lower end. Read the results visually or by using the Milksensor® Reader.

For the test lines T1 and T2 following counts:

Negative: the test line is stronger than the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits.

Positive: test line is weaker than or equal to the control line or no line appears, the milk sample contains antibiotics above the detection limits.

2.1 Configuration of the Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit test strip

The configuration of the Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit test strip is shown in Figure 1.

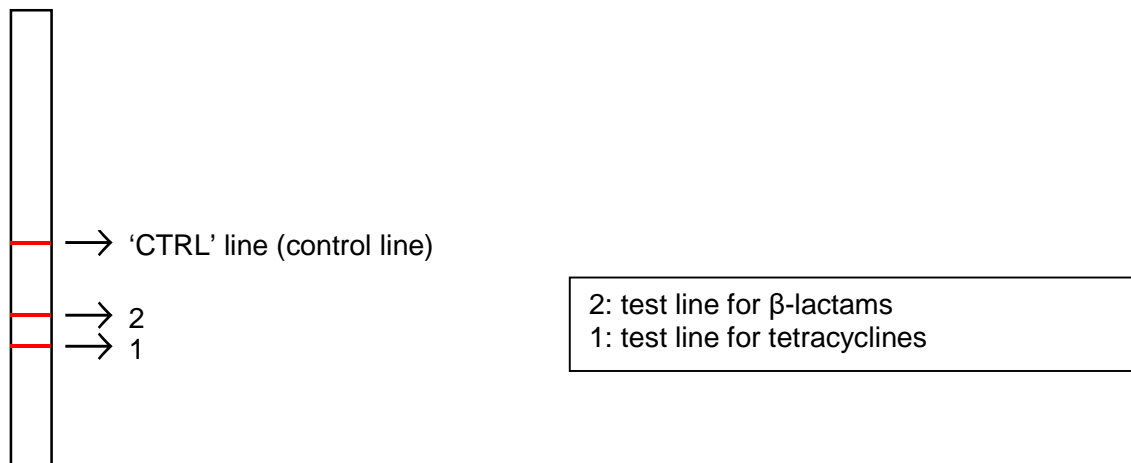


Fig. 1. Configuration of a Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit test strip.

2.2. Instrumental interpretation of the test

The Milksensor® reader is comparing the intensity of each test line with the intensity of the control (reference) line and calculates for each channel a ratio = intensity test line / intensity control line. This ratio for each test line is compared to a fixed cut-off value. The ratio cut-off levels for each test line are given in Table 1.

In this validation both 'doubt' and 'positive' are considered as positive.

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

Ratio		Interpretation
Beta-lactam channel	Tetracycline channel	
$R \leq 0.95$	$R \leq 0.95$	Positive (+)
$0.95 < R \leq 1.05$	$0.95 < R \leq 1.08$	Doubt (+/-)
$R > 1.05$	$R > 1.08$	Negative (-)



Fig. 2. Milkensor® incubator (Anon., 2021a) and reader model GIC-S1 (Anon., 2021b) for instrumental reading of the color formation on Milkensor® BT10i (β-lactams and Tetracyclines) Test Kit.

2.3 Visual interpretation of the test

Visual reading of Milkensor® BT10i (β-lactams and Tetracyclines) Test Kit is also possible. The intensity of the test line is compared to the intensity of the reference (i.e. control) line. When the color intensity of the beta-lactam or tetracycline test line is weaker than the control line or no-line appears, the sample is positive for tetracycline or beta-lactam, respectively.

Visual reading was not checked in this validation study.

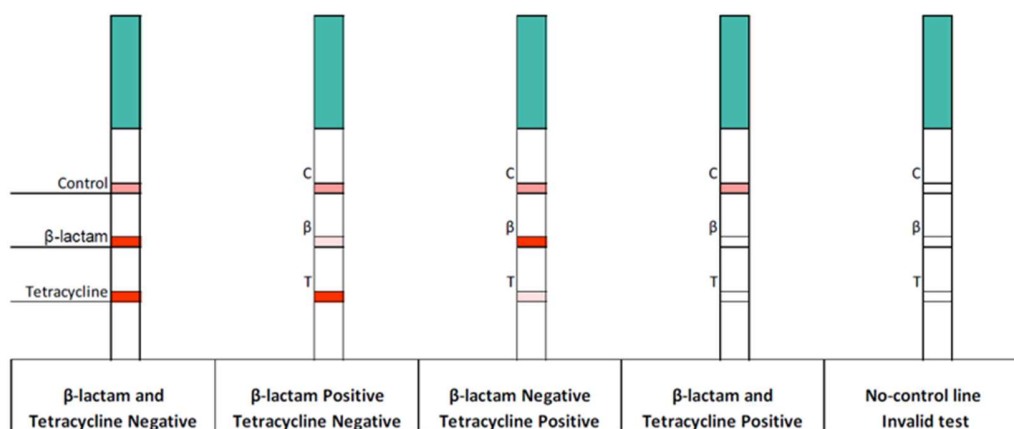


Fig. 3. Visual interpretation of the color formation on Milkensor® BT10i (β-lactams and Tetracyclines) Test Kit. (Anon., 2022)

3. Detection capability

Methods and Materials:

Spiking of antibiotic-free (blank) raw milk with β -lactams (penicillins and cephalosporins) and tetracyclines.

Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. Collected in sterile containers and kept below 4°C to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium).

The detection capability of Milksensor® BT10i (β -lactams and Tetracyclines) Test Kit was determined for a selection of β -lactams and tetracyclines mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010. Whereas for the other compounds on the list, it was tested whether they could be detection at their respective MRL level, just by testing two replicates at MRL. The spiking was performed as described in the ISO TS 23758 | IDF RM 251 (ISO|IDF, 2021). Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MRL (Table 2).

Each concentration was tested 20, 40 or 60 times in a time period of at least three days.

- Tested concentration ≤ 0.5 MRL: 20 times
- Tested concentration $>0.5 - <0.9$ MRL: 40 times
- Tested concentration $\geq 0.9 - \leq 1.0$ MRL: 60 times
- Tested concentration $>MRL$: 20 times

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

Concentration (in $\mu\text{g}/\text{kg}$)	Increment (in $\mu\text{g}/\text{kg}$)
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	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.

- Every day the following standards were also tested:
 - blank raw milk free from antimicrobials - twice
 - blank raw milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 70 µg/kg - twice
 - blank raw milk spiked with cefalonium at 3 µg/kg – twice

Detection capability tests were performed with at least 2 different lot numbers of reagents (lot 46MSi13 and 46MSi14, with as expiration date 20/12/2022 and 20/12/2022, respectively) following the manufacturer's instructions. The intensity of color formation of each test line was compared to the intensity of the control line and was interpreted by means of a Milksensor® reader model GIC-S1. The cut-off value for each test line is given in Table 1. All results (reader values) were collected in a data base.

Table 3. Standard material used in this validations study.

Compound	Origin	Product number	Lot number
4-Epichlortetracycline hydrochloride	LGC Standards	C13175500	1146697
4-Epioxytetracycline	LGC Standards	C13179000	1150794
4-Epi-tetracycline hydrochloride	TRC	T291405	1-EDT-29-1
Amoxicillin trihydrate	LGC Standards	C10242500	G1012320
Ampicillin trihydrate	LGC Standards	C10243080	G1059460
Benzylpenicillin	Sigma-Aldrich	46609	BCCB4506
Cefacetile	HPC Standards	679543	800278
Cefalexin monohydrate	LGC Standards	C11064000	G984299
Cefalonium dihydrate	LGC Standards	MM3169.00	G985467
Cefapirin sodium	LGC Standards	C11064071	G1185416
Cefazolin	LGC Standards	C11064100	G1103114
Cefoperazone sodium	LGC Standards	C11064300	G1135405
Cefquinome sulfate	LGC Standards	C11064700	G1125857
Ceftiofur hydrochloride	TRC	C244700	8-SCC-55-1
Chlortetracycline hydrochloride	Sigma-Aldrich	46133	BCBT9837
Chlortetracycline hydrochloride	Sigma-Aldrich	PHR1520	LRAC3460
Cloxacillin sodium salt monohydrate	Sigma-Aldrich	46140	BCBW1061
Desfuroylceftiofur	TRC	D289980	4-WBZ-137-3
Desacetylcephapirin sodium salt	HPC Standards	682120	805162
Dicloxacillin sodium monohydrate	LGC Standards	C12560500	G1039116
Doxycycline Hyclate	LGC Standards	C13084280	1116543
Nafcillin Sodium monohydrate	LGC Standards	C15402500	G1089700
Oxacillin sodium salt monohydrate	LGC Standards	C15755100	G1019309
Oxytetracycline hydrochloride	Sigma-Aldrich	46598	BCBZ6310
Penicillin V potassium salt	Sigma-Aldrich	46616	BCBV7003
Tetracycline hydrochloride	Sigma-Aldrich	31741	BCCC9767

Certified reference material from following different reagent suppliers was used: Sigma-Aldrich N.V. (Overijse, BE), Toronto Research Chemicals (TRC) (Ontario, CA); LGC Standards (Molsheim, FR) and HPC Standards GmbH (Borsdorf, DE). Detailed information of all standard material is given in Table 3.

Table 4. Detection capability (in µg/kg) of Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit (Mayasan SA., Istanbul, Turkey) in raw bovine milk with instrumental reading (Milksensor® reader model GIC-S1) with cut-off ratio 1.05 for the beta-lactam test line and 1.08 for the tetracycline test line. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Group	Marker residue	MR(P)L (µg/kg)	Detection capability (µg/kg)
Penicillins	Benzylpenicillin	4	2
	Ampicillin	4	2
	Amoxicillin	4	2
	Oxacillin	30	3
	Cloxacillin	30	3
	Dicloxacillin	30	(≤MRL*)
	Nafcillin	30	6
	Phenoxyethylpenicillin	-- (4) ^a	≤MRL*
Cephalosporins	Ceftiofur	100 ^b	45
	Desfuroyl ceftiofur	100 ^b	>MRL**
	Cefquinome	20	5
	Cefazolin	50	20
	Cephapirin	60 ^c	3
	Desacetylcephapirin	60 ^c	(≤MRL*)
	Cefacetile	125	8
	Cefoperazone	50	3
	Cefalexin	100	>MRL**
	Cefalonium	20	3
Tetracyclines	Tetracycline	100 ^d	30
	4-Epimer of tetracycline	100 ^d	(≤MRL*)
	Oxytetracycline	100 ^d	60
	4-Epimer of oxytetracycline	100 ^d	(≤MRL*)
	Chlortetracycline	100 ^d	125
	4-Epimer of chlortetracycline	100 ^d	>MRL**
	Doxycycline	-- ^e	≤100

Notes: (≤MRL*): Detection capability equal or below MRL, based on the testing of two replicates; exact detection capability not tested; >MRL**: Detection capability above MRL, based on the testing of two replicates; exact detection capability not tested. Bold and red font: detection capabilities are above the drug MRL. MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (actual situation). Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively.

^a: No MRL in milk, MRL based on Commission Implementing Regulation (EU) 2018/470,

^b: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur,

^c: The MRL of 60 µg/kg in milk is applied on the sum of cephalapirin and desacetylcephapirin,

^d: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer,

^e: No MRL in milk, not for use in animals from which milk is produced for human consumption.

Results:

A summary of Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit detection capabilities is given in Table 4.

Discussion:

The Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit is capable to detect all residues of β-lactams (penicillins and cephalosporins) and tetracyclines present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010). For those β-lactams for which the 95% detection capability was determined, all can be detected at least in 95% of the replicates at their respective MRL. For the other β-lactams, testing of two replicates shows that all are detected at their respective MRL concentration except for desfuroylceftiofur and cefalexin. All tetracyclines can be detected at least in 95% of the replicates at their respective MRL, except for chlortetracycline (ccβ= 125 µg/kg; MRL = 100 µg/kg). It is worth noting that the detection capability of chlortetracycline was determined with two different reference materials. The detection capability of their 4-epimers was not determined, but they are detectable at MRL level, except for the 4-epimer of chlortetracycline. The 95% detection capability of doxycycline, not for use in animals from which milk is produced for human consumption, was not determined, but the compound can be detected at least below 100 µg/kg.

The test is fulfilling the current acceptance criteria (in force until 30 June 2023) and could be approved by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) as test used by the Belgian dairy companies to check incoming milk on the presence of β-lactam residues (Anon., 2021c). But the test is not fulfilling the more strict FASF acceptance criteria coming into force from July 1, 2023 on (Anon., 2021c).

4. Stability of reagents – daily control samples

Methods and material:

The following control samples were analyzed daily (twice) with Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk) – both fresh milk and frozen milk samples
- Raw milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 70 µg/kg
- Raw milk spiked with cefalonium at 3 µg/kg.

Also the negative and positive control samples included in the kit were analysed daily (lotnumber and expiry date equal to reagents).

For the negative control, take 200 µl of HPLC-water and put into the negative control microwells. Mix homogenously by pipetting up and down. Then transfer the mixture into a regular microwell and mix homogenously with the reagent.

For the positive control microwells, take 200 µl of negative milk sample and put into the microwell and mix homogenously. Transfer the mixture into a regular microwell and mix homogenously with the reagent.

Results:

The results of the daily control samples are presented in Figure 4 and 5. A summary is provided in Table 5. The results of the daily negative and positive control samples included in the kit are presented in Figure 6 and Table 5.

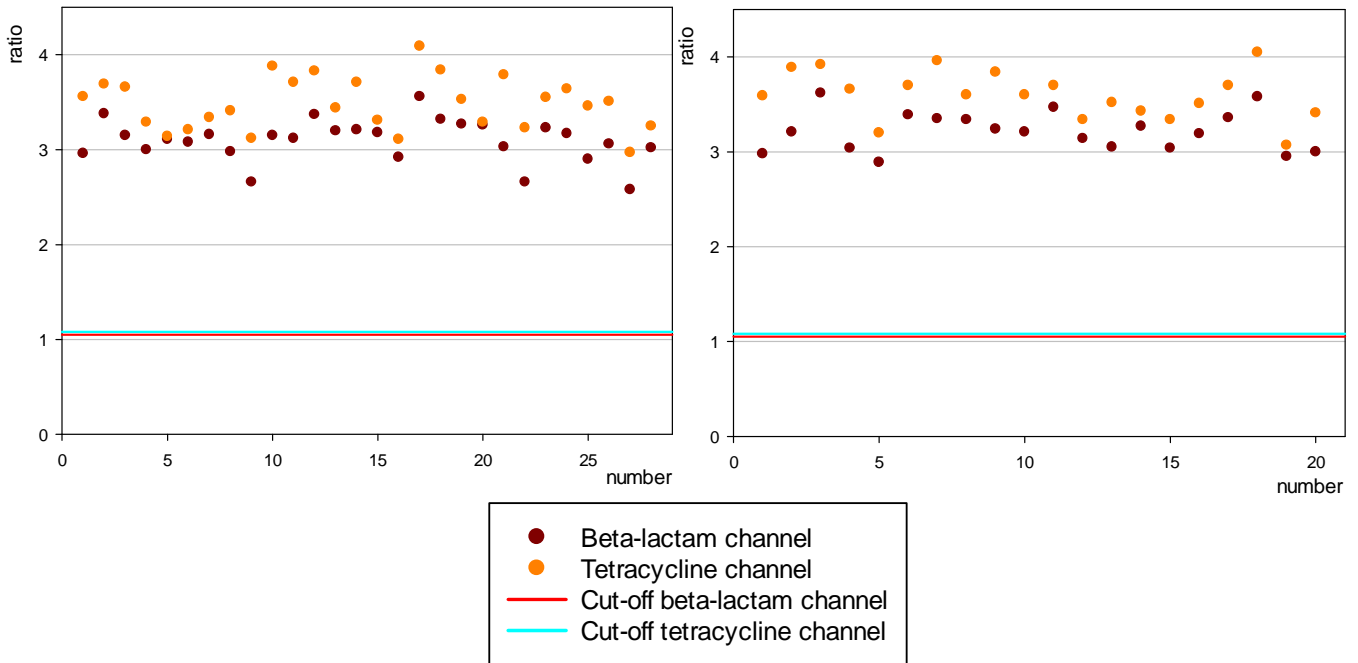


Fig. 4. Milksensor® BT10i (β -lactams and Tetracyclines) Test Kit results (ratio) for the blank (left: fresh blank samples, right: frozen blank samples). Beta: β -lactam; Tetra: tetracycline.

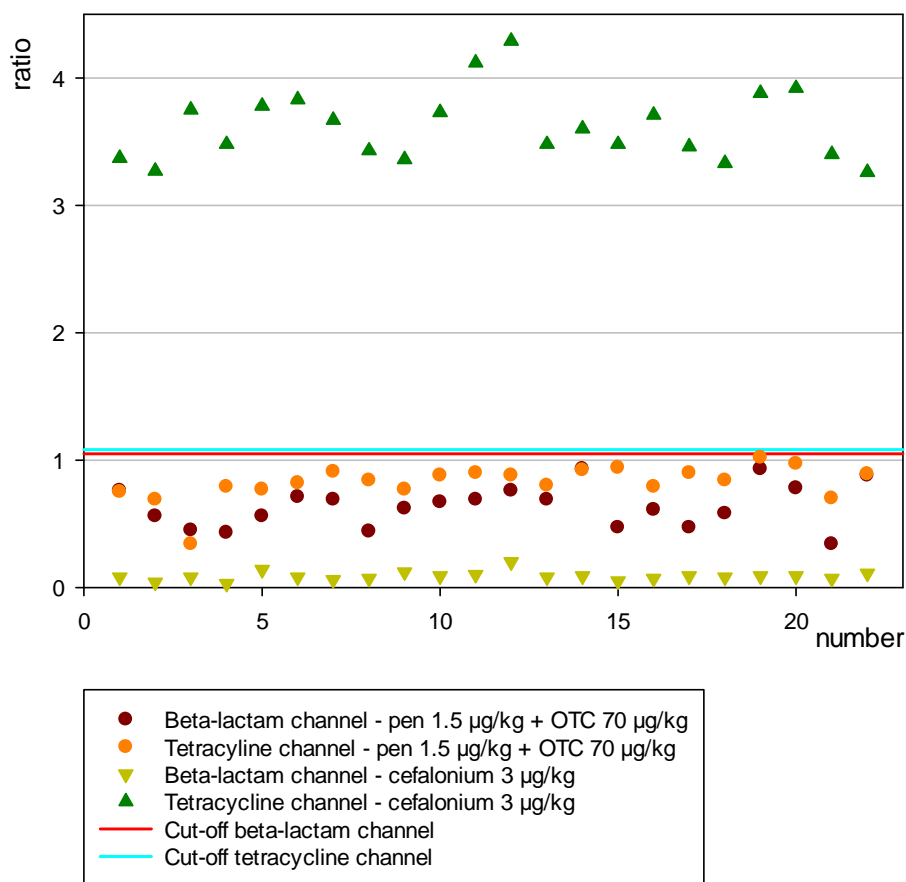


Fig. 5. Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit results (ratio) for the samples spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 70 µg/kg and for the samples spiked with cefalonium at 3 µg/kg. Beta: β-lactam; Tetra: tetracycline.

Table 5. Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit results (ratio values) for the daily standards and negative and positive control samples.

Standard	Tetracycline-channel				Beta-lactam channel			
	mean	min	max	s _r	mean	min	max	s _r
Daily standard - Blank milk								
Fresh milk	3.10	2.58	3.56	0.22	3.48	2.97	4.09	0.28
Frozen milk	3.21	2.89	3.62	0.21	3.60	3.07	4.05	0.26
Daily standard - Milk spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 70 µg/kg.								
	0.64	0.34	0.93	0.17	0.82	0.34	1.02	0.14
Daily standard - Milk spiked with cefalonium at 3 µg/kg								
	0.09	0.03	0.20	0.03	3.62	3.26	4.29	0.28
Controls included in kit								
Negative	2.60	1.93	2.95	0.28	3.45	2.95	4.03	0.31
Positive	0.12	0.05	0.53	0.12	0.40	0.24	0.78	0.15

Notes: s_r: standard deviation; mean: mean ratio; min: lowest ratio; max: highest ratio.

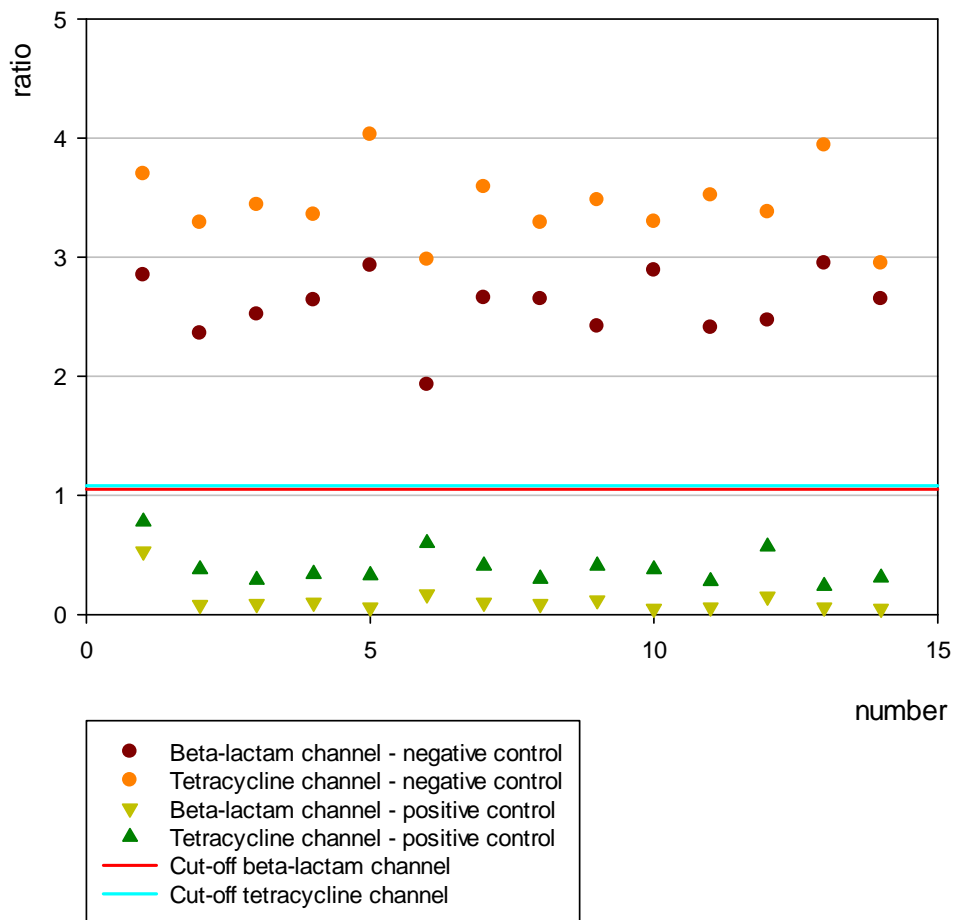


Fig. 6. Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit results (ratio) for the negative and positive control samples included in the kit. Beta: β-lactam; Tetra: tetracycline.

Discussion:

In general very stable ratio values were obtained for daily control samples with the Milksensor® BT10i (β-lactams and Tetracyclines) Test Kit reagents over the test period on all tests lines. In general, correct values were obtained for the different daily standards: all blank milk standards (fresh and frozen) gave a negative result on all channels.

For the milk samples spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 70 µg/kg, and those spiked with cefalonium at 3 µg/kg, all tested positive on the respective test channels. The daily negative and positive control samples included in the kit gave correct results at all times. Negative control samples tested always as negative, positive control samples tested always as positive.

5. Reliability of the instrumentation

- During this validation study, no problems with the reader system not incubator were encountered.
- When removing the sample pad of the strip people have to take care that no sample pad residues are left behind, as even the littlest residue might give false results.
- The negative test control does not dissolve very well in HPLC-water.
- The cups break easily when you need to separate them.
- One tube contained a test strip which was much larger (wider) than the others, and could not be read with a reader. For visual interpretation, this did not make any difference.

6. Interlaboratory testing

Methods and material:

T&V-ILVO organizes twice a year a national ring trial for the (Belgian) dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. In the next ring trail, which will be organised in spring 2022, Milksensor® BT10i (β -lactams and Tetracyclines) Test Kit will be integrated as rapid test.

7. Final conclusions

Results of this validation show that Milksensor® BT10i (β -lactams and Tetracyclines) Test Kit is a reliable test for screening of raw cows' milk for residues of β -lactam (penicillins and cephalosporins) and tetracycline antibiotics below their MRL (EU-Regulation 37/2010 and amendments). Of compounds for which the 95% detection capability was determined, only chlortetracycline was not detected in 95% of the replicates below its' MRL (cc β = 125 μ g/kg; MRL = 100 μ g/kg). Of several compounds the 95% detection capability was not determined (see Table 4), but testing of two replicates indicate that the test is able to detect the compounds at MRL, except for cefalexin, desfurouylceftiofur and the 4-epimer of chlortetracycline for which both replicates were not detected at their respective MRL.

No selectivity/specificity tests, nor robustness tests were performed in this short validation.

ACKNOWLEDGEMENT

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