# BT Scan

Immunochromatographic test
for β-lactams and tetracyclines in milk

## TECHNICAL REPORT



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## 1. INTRODUCTION

The use of antimicrobials due to preventive or curative treatments is a common practice in livestock animals. Antibiotics from β-lactams and tetracyclines families are the most widely used in dairy industry. The administration of these drugs may lead to the presence of violative levels of antimicrobial residues in edible products of animals. Concerns about the presence of drug residues in milk are due to technological problems in fermented dairy products, toxicity and potential allergic reactions in sensitised individuals. Besides, according to WHO, antimicrobial resistances are one of the most serious threats to global public health over the world.

The European authorities have established a legislation framework setting maximum residue limits in animal foodstuffs (Commission Regulation No. 37/2010) (1), monitoring plans (Council Directive 96/23/EC) (2) and analytical requirements for the methods to be used (Commission Decision 2002/657/EC) (3).

## 2. PRINCIPLE AND SCOPE

BT Scan is a qualitative immunochromatographic test, in strip format, to detect β-lactams (β) and tetracyclines (T) in milk.

It is a competitive test consisting in a strip placed in a cassette that contains specific receptors and antibodies. The strips have 3 lines: the upper line is the control line (C), the central line is for β-lactams (β) and the lower line for tetracyclines (T).

If the sample is free from these antibiotics, the receptors will bind to its specific line of the strip when the liquid runs through the strip, and intense red lines will appear. When some antibiotic (β-lactam or tetracycline) is present in the sample, it will bind the receptors which then will not be able to bind to the specific line in the strip, inhibiting the appearance of the red colour of the corresponding test line, or decreasing its intensity.

The BT Scan test can be used with raw, heat treated and powder cow milk and raw milk from different species: cow, buffalo, sheep and goat.

## 3. PROCEDURE

Samples do not need any preparation. IRIS reader has to be switch on 10 min before start an assay to get the suitable assay temperature. Insert a cassette in the IRIS reader, then add 100 µL of milk sample into the cassette cavity. After pressing "Run test", it incubates for 6 minutes and results are read afterwards. Results are shown in the IRIS reader screen or the smartphone IRIS app.

1. Tap "Automatic assay" and scan the QR code
***Selecciona "Automatic assay" y escanea el código QR***


2. Insert the test in IRIS
***Inserta el test en IRIS***


3. Add 100 µL of milk
***Añade 100 µL de leche***


4. Tap "Run Test"
***Selecciona "Run Test"***

5. Check results
***Comprueba los resultados***

## 4. INTERPRETATION OF RESULTS

When the reading is done, in the 'result screen', two ratio Test Line/Control Line values will be displayed. The first one is for β-lactams (BL/CL) and the second one for tetracyclines (TL/CL).

If the ratio is below or equal to 1.0, the sample is positive for the respective family of antibiotics and if it is above 1.0, the result is negative. To easy the interpretation the positive/negative designation is also displayed in the 'result screen'.

## 5. VALIDATION AND TECHNICAL SPECIFICATIONS

### 5.1. Detection Capabilities (CCβ) and Limits of Detection (LOD)

The system sensitivity was evaluated in two steps. First, limits of detection (LOD) of 16 molecules belonging to the β-lactams and tetracyclines families were determined. Then, the

detection capability (CCβ) was determined for 5 of those molecules as the most representative of each family.

The limits of detection for the 15 molecules were determined in bulk raw milk following a procedure adapted from the ISO 13969:2003. According to these guidelines, at least three to five replicates should be analysed for each substance/level combination when a test is evaluated with an objective reading. The limit of detection was considered as the concentration at which 100% of replicates were positive (3-5 replicates). Table 1 summarizes the limits of detection in raw milk for 16 antimicrobials. Most of the antimicrobials (14 of 15) showed detection limits at or below the MRL.

Table 1. Limits of detection (µg/L) of BT Scan in cow milk.

| | | MRL (µg/Kg, ppb) | LOD (µg/Kg, ppb) |
|---|---|---|---|
| **β-lactams** | Amoxicillin | 4 | 3 |
| | Ampicillin | 4 | 2 |
| | Penicillin | 4 | 2 |
| | Cloxacillin | 30 | 5 |
| | Oxacillin | 5 | 5 |
| | Cephaperazon | 50 | 5 |
| | Cefquinome | 20 | 10 |
| | Nafcillin | 30 | 15 |
| | Cephapirin | 60 | 8 |
| | Cephalexin | 100 | > 600 |
| | Cephalonium | 20 | 4 |
| | Ceftiofur | 100 | 100 |
| **Tetracyclines** | Oxytetracycline | 100 | 60 |
| | Tetracycline | 100 | 40 |
| | Chlortetracycline | 100 | 40 |

Limits of detection in raw milk of goat and sheep were also tested for some antimicrobials. LODs are shown in table 2.

Table 2. Limits of detection (µg/L) of BT Scan in goat and sheep milk.

| | | MRL (µg/Kg, ppb) | GOAT LOD (µg/Kg, ppb) | SHEEP LOD (µg/Kg, ppb) |
|---|---|---|---|---|
| **β-lactams** | Amoxicillin | 4 | 3 | 3 - 4 |
| **Tetracyclines** | Oxytetracycline | 100 | 100 | 100 |

Detection capability (CCβ) values were determined in bulk raw milk according to the CRL guidelines (4). According to these guidelines, the number of replicates to be evaluated depends on the closeness of the detection capability to the regulatory limit. Thus, each molecule has to be tested 20, 40 or 60 times in different days, with different samples and different antibiotic preparations and tests batches. If the detection limit of the method is equal to half the MRL or lower, a maximum of one false-compliant result of 20 spiked samples at such level is enough to

ensure that CCβ complies with the MRL. However, if the detection limit ranges between 50 and 90% of the MRL, at least 40 spiked samples at such level have to be analysed with no more than two false-compliant results. When the detection limit approaches the MRL, 60 spiked samples will be necessary (with no more than three false-compliant results) to demonstrate that CCβ is fit for the purpose. Finally, when the detection limit is higher than the MRL, 20 spiked samples will be necessary to analyse.

Table 3. Detection Capabilities (µg/l) of BT Scan in raw cow milk.

| Inhibitor | MRL (µg/L, ppb) | CCβ (µg/L, ppb) | No. positive/ No. samples |
|---|---|---|---|
| Amoxicillin | 4 | 3 | 47/47 |
| Cloxacillin | 30 | 10 | 23/23 |
| Cefquinome | 20 | 15 | 43/45 |
| Ceftiofur | 100 | 80 | 40/40 |
| Oxytetracycline | 100 | 60 | 44/46 |

### 5.2. False positives

To determine the false positive ratio of the test, 250 cow milk samples were analysed. Three positive samples for beta-lactams were detected very close to the cut-off value. These samples were also analysed with *Eclipse*, a microbial inhibition method, obtaining a negative result for all of them. However, according to the difference in detection limits of the rapid test and of Eclipse and the closeness to the cut-off value, samples could contain a beta-lactam at a concentration below the LOD of Eclipse or Cefquinoma that it is not detected with Eclipse at the MRL level.

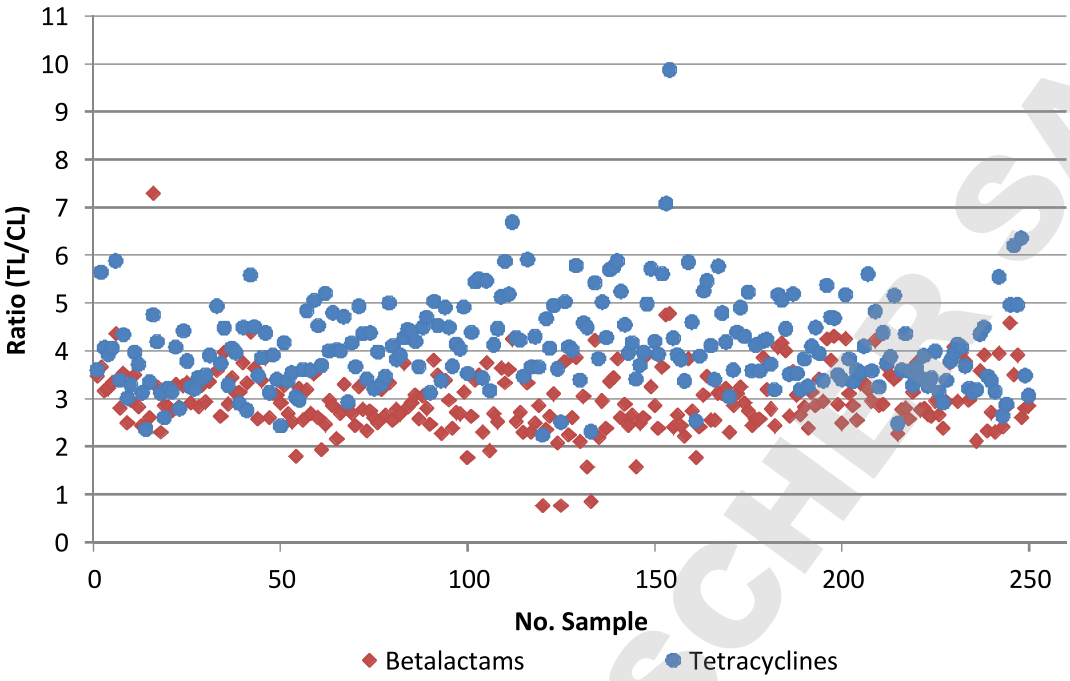


Figure 1. Results obtained for the 250 raw milk samples from individual cows.

### 5.3. Ruggedness

To determine the ruggedness of the test, the influence of sample volume, milk sample initial temperature and pH, incubation temperature, incubation time and the delayed of the reading time on the false-positive rate were evaluated. The effect on sensitivity was focused on one representative molecule of each family; oxytetracycline for TC and amoxicillin for BL, both at its CCβ concentration.

To perform the assays in the following sections, five negative milk samples and five milk samples spiked with 2 different antibiotics were prepared and analysed following the procedure described in section 3.

#### 5.3.1. Effect of sample volume

The effect of the sample volume was evaluated by varying up to 10% the nominal volume (Table 4). All negative samples gave values above 1.0 (negative result), while the spiked samples gave a result below 1.0 (positive result), regardless the applied volume. Therefore, neither false positives nor false negatives were obtained by varying up to 10% the volume of sample.

Table 4. Effect of the sample volume on the performance of BT Scan. Results are presented as average ratio values from the 5 replicates.

| | | 90ul | 100ul | 110ul |
|---|---|---|---|---|
| **Ratio TC/CL** | 0ppb | 3.7 - | 3.4 - | 3.2 - |
| | 60 ppb Oxytetracycline | 0.4 + | 0.4 + | 0.3 + |

| | | | | |
|---|---|---|---|---|
| **Ratio BL/CL** | 0ppb | 3.5 - | 3.2 - | 3.4 - |
| | 3 ppb Amoxicillin | 0.6 + | 0.6 + | 0.6 + |

### 5.3.2. Effect of the initial sample temperature

Usually, it is recommended to take samples out of refrigeration some minutes before the analysis. For this reason, the effect of the initial temperature of the sample was studied by comparing test results when samples are tempered at room temperature before analysis and when they are analysed right after taking them out of refrigeration.

Average Ratio TL/CL (Test Line/Control Line) values are shown in table 5. All negative samples gave negative results (ratio above 1.0), while the spiked samples gave a result below the cut-off, regardless the initial sample temperature. In this way, no false-positive result was found by analysing the sample fresh out of refrigeration.

Table 5. Effect of the initial sample temperature on the performance of BT Scan. Results are presented as average ratio values from the 5 replicates.

| | | **Room Temperature (22°C)** | **Cold (4°C)** |
|---|---|---|---|
| **Ratio TC/CL** | 0ppb | 3.3 - | 3.9 - |
| | 60ppb Oxytetracycline | 0.4 + | 0.5 + |
| **Ratio BL/CL** | 0ppb | 3.7 - | 4.2 - |
| | 3ppb Amoxicillin | 0.9 + | 0.7 + |

### 5.3.3. Effect of milk sample pH

When milk is spoiled, it is usual that it becomes more acidic and the pH gets lower, below its usual pH of 6.7. The effect of pH sample was evaluated by varying the usual 6.7 pH of milk, in a 0.3 range (Table 6). All negative samples gave values above 1.0 (negative result), regardless the pH, so no false-positive results were found.

In the spiked samples, the positive result is not altered when the pH is increased. When pH decreases below the optimal values for milk, samples with 3 ppb of amoxicillin gave negative results.

Table 6. Effect of the milk sample pH on the performance of BT Scan. Results are presented as average ratio values from the 5 replicates.

| | | **pH 6.4** | **pH 6.7** | **pH 7.0** |
|---|---|---|---|---|
| **Ratio TC/CL** | 0ppb | 4.0 - | 3.3 - | 3.9 - |
| | 60ppb Oxytetracycline | 0.3 + | 0.4 + | 0.6 + |
| **Ratio BL/CL** | 0ppb | 4.0 - | 3.7 - | 4.4 - |
| | 3ppb Amoxicillin | 1.2 - | 0.9 + | 0.8 + |

### 5.3.4. Effect of incubation temperature

The optimal incubation temperature for BT Scan is 40°C and the effect of modifying the incubation temperature was tested. It was compared the optimal condition with analyses of the same samples with the incubation steps at room temperature (ca. 23°C) and 45°C.

All negative samples gave values above 1.0 (negative result), so no false-positive results were found.

In the spiked samples, the result is not altered and positive results are obtained, both at room temperature and at 45°C. However, when the assay is performed at room temperature, the ratio values are closer to the cut-off level.

Table 7. Effect of the incubation temperature on the performance of BT Scan. Results are presented as average ratio values from the 5 replicates.

| | | Room Temp. (23°C) | 40°C | 45°C |
|---|---|---|---|---|
| **Ratio TC/CL** | 0ppb | 2.2 - | 3.2 - | 3.4 - |
| | 60ppb Oxytetracycline | 0.2 + | 0.4 + | 0.5 + |
| **Ratio BL/CL** | 0ppb | 2.4 - | 3.3 - | 3.0 - |
| | 3ppb Amoxicillin | 1.0 + | 0.6 + | 0.5 + |

### 5.3.5. Effect of incubation time

The optimal incubation time is 6 minutes at 40°C. The effect of this parameter was tested by varying up to 1 minute the time and comparing the results with those obtained in optimal conditions.

In all cases negative results were obtained in the non-spiked samples and no appreciable changes were found for oxytetracycline and amoxicillin samples.

Table 8. Effect of the incubation time on the performance of BT Scan. Results are presented as average ratio values from the 5 replicates.

| | | 5 min | 6 min | 7 min |
|---|---|---|---|---|
| **Ratio TC/CL** | 0ppb | 3.8 - | 3.4 - | 3.2 - |
| | 60ppb Oxytetracycline | 0.4 + | 0.4 + | 0.4 + |
| **Ratio BL/CL** | 0ppb | 3.5 - | 3.2 - | 3.4 - |
| | 3ppb Amoxicillin | 0.8 + | 0.6 + | 0.6 + |

In conclusion, false-positive and false-negative results were not found by varying up to 1 minute the incubation time.

### 5.3.6. Effect of delaying reading time

When time passes after the end of the test, both the test and control lines, become more intense. Because of this, the ratios can vary if the results are not read immediately after the test is finished.

Ratios TC/CL and BL/CL were monitored at 0, 1, 5 and 15 minutes after the end of the assay for one negative milk sample and one positive milk sample with both antimicrobials (oxytetracycline and amoxicillin). In both cases five replicates of each sample were analysed. In table 9, averaged ratio values (test line/control line) at each time are shown.

Table 9. Effect of delaying the reading time on the performance of BT Scan.

| | | 0 min | 1 min | 5 min | 15 min |
|---|---|---|---|---|---|
| **0 ppb** | Ratio TC/CL | 3.2 - | 3.1 - | 2.9 - | 2.6 - |
| | Ratio BL/CL | 3.3 - | 3.1 - | 2.7 - | 2.3 - |
| **60 ppb Oxytetracycline + 3 ppb Amoxicillin** | Ratio TC/CL | 0.4 + | 0.4 + | 0.3 + | 0.3 + |
| | Ratio BL/CL | 0.6 + | 0.6 + | 0.5 + | 0.4 + |

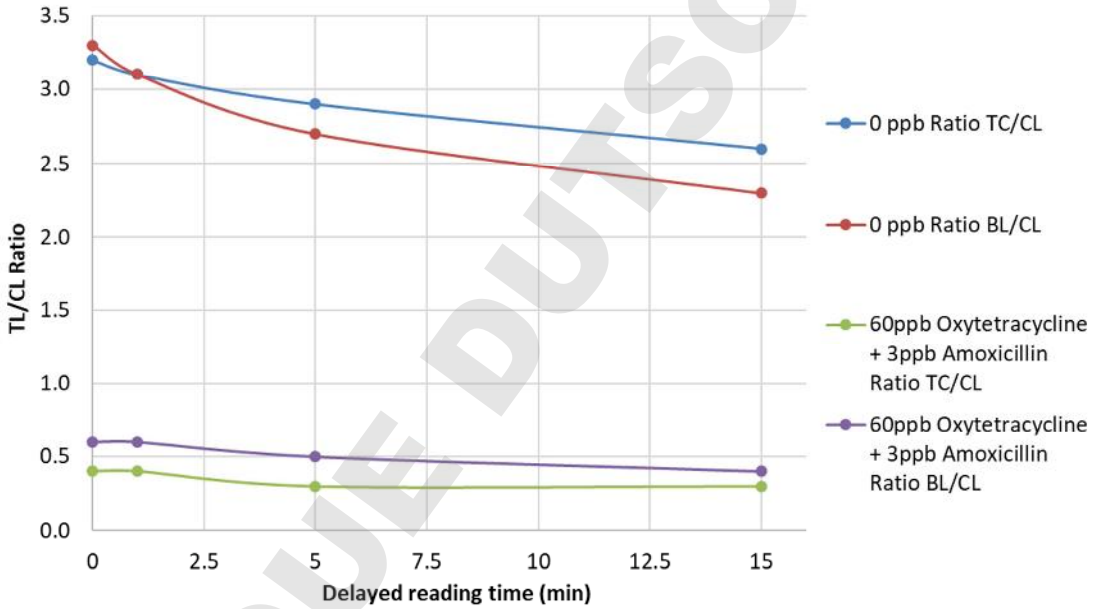


Figure 2. Effect of delaying the reading time on the performance of BT Scan.

A general decrease in ratios was found although false-positive and false-negative were not found.

Using IRIS reader, the assay is automatic and there is no risk of reading delays.

### 5.4. Specificity

Specificity of BT Scan to different antimicrobials from families other than β-lactams and tetracyclines was determined in raw cow milk (Table 10). Concentration of antimicrobials 10 to 200 higher than the MRL were detected as negative. These results indicate that BT Scan detect specifically β-lactams and tetracyclines at MRL but does not detect other antimicrobials.

Table 10. Specificity of BT Scan against antimicrobials other than β-lactams and tetracyclines

| Antibiotic (n=2) | Family | MRL (ppb) | Tested concentration | RESULT TC | RESULT BL |
|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| Negative milk sample | - | - | 0ppb | | NEGATIVE | NEGATIVE |
| Tylosin | Macrolide | 50 | 10 ppm | (MRLx200) | NEGATIVE | NEGATIVE |
| | | | 1 ppm | (MRLx20) | NEGATIVE | NEGATIVE |
| Gentamycin | Aminoglycoside | 200 | 20 ppm | (MRLx100) | NEGATIVE | NEGATIVE |
| | | | 2 ppm | (MRLx10) | NEGATIVE | NEGATIVE |
| Enrofloxacin | Quinolone | 100 | 10 ppm | (MRLx100) | NEGATIVE | NEGATIVE |
| | | | 1 ppm | (MRLx10) | NEGATIVE | NEGATIVE |
| Lincomycin | Lincosamide | 150 | 150 ppm | (MRLx100) | NEGATIVE | NEGATIVE |
| | | | 1.5 ppm | (MRLx10) | NEGATIVE | NEGATIVE |
| Sulfadiazine | Sulfamide | 100 | 10 ppm | (MRLx100) | NEGATIVE | NEGATIVE |
| | | | 1 ppm | (MRLx10) | NEGATIVE | NEGATIVE |
| Sulfatiazol | Sulfamide | 100 | 10 ppm | (MRLx100) | NEGATIVE | NEGATIVE |
| | | | 1 ppm | (MRLx10) | NEGATIVE | NEGATIVE |
| Dapsone | Sulfone | - | 50 ppb | | NEGATIVE | NEGATIVE |
| Chloramphenicol | Amphenicol | - | 50 ppb | | NEGATIVE | NEGATIVE |

## 6. REFERENCES

(1) 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. Official Journal of European Union 2010 L15:1–72.
(2) Council Directive 96/23/EC monitoring plans…
(3) Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and interpretation of results. Official Journal of European Communities L 221:8–36.
(4) Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer of method). Community Reference Laboratories Residues. Document of 21 January 2010. pp. 1–18.

For further information consult BT Scan kit instructions.