• *Pseudomonas*
• *Enterococcus and Enterobacteriacea*
• *Streptococcus sp*
• *Bacillus and Clostridium*
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1. PRINCIPLE OF THE TEST

The qPCR test with Cat. No. TBC4 can be used for screening of 384 milk samples for four bacteria groups:

- **TBC 4**
- *Pseudomonas*
- *Enterococcus* and *Enterobacteriacea*
- *Streptococcus sp*
- *Bacillus* and *Clostridium*

TBC 4 is the name of a qPCR assay for investigating some bacteria involved in Total Bacterial Count (TBC) using a fast, sensitive, and easy method, to test for detection of four groups of pathogens causing high bactocount. TBC 4 testing involves an easy-to-do DNA extraction from 0.5 mL milk samples or swabs followed by a fast qPCR reaction. Test results can be acquired in 3 hours with very little hands on time. The TBC 4 tests can be used on bulk tank milk or individual milk samples or swabs. The samples can be preserved or non-preserved.

The qPCR test use approximately 1/12 of the purified DNA as template. The remaining DNA can be used for testing with other Mastit 4 variants or stored at -20°C for later use.

The qPCR reaction contains four sets of primers and fluorescence probes for specific detection of four bacteria groups. A fifth set of primers and probe detects an internal amplification control (IAC).

The qPCR instrument generates an amplification plot and Ct-values (Cycle at threshold). Each of the four bacteria probes emit a specific light (color) enabling the identification of bacteria present in the sample.

The tests use the standard 96 well plate format and ready-to-use reaction mixes. The test requires only pipettes, a vacuum aspirator, a centrifuge, a thermal heating block and a qPCR instrument.
2. KIT COMPONENTS AND STORAGE

The TBC 4 kit contains material for testing 4x96 samples. One kit contains two boxes, one for storage at room temperature and one for storage at -20°C.

**Box for storage at room temperature contains:**
- Four sealed Deep Well Plates each with 96 wells containing a solution.
- Four bottles with Wash Buffer, 100 mL each.
- Four 96 Well Plates (0.2 mL clear tubes) with caps.
- 12 pieces of sealing tapes for Deep Well plates.
- Four 96 cap mats for the qPCR 96 Well Plates.

**Box for storage at -20°C contains:**
- Two bottles with Lysis Buffer-I, 10800 uL each.
- Two yellow capped tubes with Mix I Additive for Lysis Buffer-I, 1200 µL each.
- Four sealed 96 Well Plates (0.2 mL white tubes) with qPCR Master Mix.

3. REQUIRED EQUIPMENT

- Centrifuge for 96 well plates, centrifuge must be capable of running at 5000xg at 37°C
- Pipettes and sterile filter tips
- Incubation oven at 37°C
- Plate washer / vacuum system to aspirate supernatant from Deep Well Plates (Biorad DW40 can be used)
- PCR instrument for 37°C and 95°C incubations during lysis. Alternatively, the qPCR machine can be used.
- qPCR instrument, Stratagene Mx3005 with filters for ATTO (440nm-492nm), FAM (492nm-516nm), HEX (535nm-555nm), ROX (585nm-610nm), CY5 (635nm-665nm).
4. PROTOCOL

DNA purification
When extracting from DNA Diagnostic FLOQSwabs, please see section 5 for Swab addendum instead of step 1 and 2.

1. Spin a 96 Deep Well Plate 1 min at 1000 g and remove seal. Add 0.5 ml milk sample to each of the wells. Cover wells with sealing tape.

2. Incubate the Deep Well Plate at 37°C for 10 min.

3. Spin Deep Well Plate with milk at 37°C and 5000xg for 5 min. and discard sealing tape. Remove supernatants from the top with tips connected to a vacuum system. Be careful not to touch or remove the bacterial pellets.

4. Add 1 mL Wash Buffer to each tube. Cover with sealing tape.

5. Spin at 5000xg and at 37°C for 5 min. and discard sealing tape. Remove supernatants from the top with tips connected to a vacuum system. Be careful not to touch or remove the bacterial pellets. It is important to remove the supernatant completely.

6. Prepare fresh Lysis-I Mix by adding 6 µL Mix I additive to 54 µL Lysis buffer-I and mix.

<table>
<thead>
<tr>
<th>Lysis-I Mix</th>
<th>1 reaction</th>
<th>9 reaction</th>
<th>100 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer-I</td>
<td>54 µL</td>
<td>486 µL</td>
<td>5400 µL</td>
</tr>
<tr>
<td>Mix I additive</td>
<td>6 µL</td>
<td>54 µL</td>
<td>600 µL</td>
</tr>
<tr>
<td>TOTAL</td>
<td>60 µL</td>
<td>540 µL</td>
<td>6000 µL</td>
</tr>
</tbody>
</table>

7. Add 60 µL Lysis-I Mix, to each pellet and cover with sealing tape. Vortex the Deep Well plate 10 seconds and spin 20 seconds at 1000xg to bring Lysis mix to the tube bottoms.

8. Remove caps from 96 well plate with clear 0.2 mL tubes.

9. Use an 8-channel pipette with filter tips for transfer of 60 µL from each well of the Deep Well Plate into the corresponding tubes in the 96 well plate (clear 0.2 mL). Close tubes with the caps. Avoid cross contamination. Use one new tip per tube.

10. Incubate the 96 well plate at 37°C for 20 min.

11. Incubate the 96 well plate at 95°C for 15 min.
12. Cool the 96 well plate on ice for 5 min
   Note: The incubation at 37°C and 95°C can be done using a PCR instrument programmed:
   37°C for 20 min. → 95°C for 15 min. → 4°C for 5 min.

13. Centrifuge the 96 well plate at 5000xg for 5 min. at room temperature. Note it is important
    to use a 96 tube support for the 96 well plate during centrifugation.

14. Carefully remove the caps from the 96 well plate. Use an 8-channel pipette with filter tips to
    transfer 5 µL of each aqueous phase directly to the corresponding tubes of the 96 well plate
    with qPCR Master Mix in step 18.

15. The remaining purified DNA can be stored at -20°C for long time storage.

qPCR analysis
16. Take a 96 Well Plate with qPCR Master Mix (white 0.2 mL tubes) from -20°C, place on ice
    for five minutes and spin 20 seconds at 1000xg to bring qPCR Master Mix to the tube
    bottoms.

17. Discard the seal from the 96 Well Plate with qPCR Master Mix and place the 96 well plate
    on ice.

18. Transfer 5 µL purified DNA from step 14 to each of the corresponding tubes in the 96 well
    plate containing 15 µL qPCR Master Mix.
    Note: It is important to keep Master Mix on ice while loading template and to run the qPCR
    within 15 min.

19. Carefully close the qPCR tubes with a new optical lid. Spin the tubes briefly before transfer
    of the 96 Well Plate qPCR reactions to the qPCR instrument. Note it is important to keep
    the optical lids clean.

Instrument guideline settings for the MX3005P
20. Filter Gain Settings:

<table>
<thead>
<tr>
<th>ATTO</th>
<th>FAM</th>
<th>HEX</th>
<th>ROX</th>
<th>CY5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x</td>
<td>2x</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
</tr>
</tbody>
</table>

Note: If the raw data signal (R) is lower than 5000 for a color at the cycles 7-11 then
increase the filter gain setting during the next runs resulting in a raw data signal (R) above
5000.

The guidelines for setting baseline and threshold should be checked manually to ensure that
threshold lines are in the exponentially area of the amplification plot and above the background.

21. Threshold Fluorescence. Select “Background Based Threshold” to cycles 7-11 and set the
    “Sigma multiplier” to 10.
    To avoid bias of the Threshold wells including amplifications curve with Ct<15 is flag out.
The threshold is then looked based on the rest of the sample and the sample with \( Ct < 15 \) are put into the analysis again.

22. **Baseline Correction.** Select “Adaptive baseline”.

23. Activate the following filters: ATTO, FAM, HEX, ROX, CY5.

24. Run qPCR with the following cycling parameters:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>1 min.</td>
<td>1</td>
<td>Heat activation</td>
</tr>
<tr>
<td>95°C</td>
<td>5 sec.</td>
<td>40</td>
<td>Amplification</td>
</tr>
<tr>
<td>60°C</td>
<td>25 sec.*</td>
<td></td>
<td>* Read fluorescence for ATTO, FAM, HEX, ROX, CY5 at the end of each extension step.</td>
</tr>
</tbody>
</table>

5. **Swab addendum**

1. Add 750uL Swab Elution Buffer in a 2mL deepwell (or 2mL tube).
   Remove the swab from the tube. If using deepwell plates, also remove the swab tip and plastic stick from the swab lid. Insert the swab tip into the deepwell (or 2mL tube) containing Swab Elution Buffer.

2. Incubate 10 – 120 minutes at 37 °C.
   Stir deepwell plate with swabs on 96-well vortexer at 500 rpm for 2 minutes. Make sure that the liquid does not spill out of the wells.
   If using single tubes, stir the swab around in the liquid and try to press the swab against the well wall to press out liquid.
   Remove and discard the swab.

6. **INTERPRETATION OF TEST RESULTS**

Generate amplification plots showing:

- Thermal cycles on the X-axis.
- Background subtracted fluorescence (dR) on the Y-axis.
- \( Ct \) values are calculated automatically by the instrument. The Stratagene MxPro software should be set to Adaptive Baseline Correction and Background-based Threshold using cycle 7-11 and a sigma multiplier of 10.

- **Samples with \( Ct \) values below 37** are considered as true positive.
- **Samples with \( Ct \) values above 37** should be consider negative.

An IAC amplification signal in the ATTO filter should be present in all reactions. The IAC \( Ct \)-value must be maximum 32 for the test to be valid. \( Ct \) above 32 is only accepted when the reaction is highly positive (low \( Ct \)-value) for one of the bacteria in the test.
## INTERPRETATION TABLE

<table>
<thead>
<tr>
<th>Master mix</th>
<th>qPCR target</th>
<th>Filter</th>
<th>Positive Ct</th>
<th>Negative Ct</th>
<th>Retest Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBC 4</td>
<td><em>Bacillus and Clostridium</em></td>
<td>CY5</td>
<td>≤37</td>
<td>None or &gt;37</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus sp</em></td>
<td>ROX</td>
<td>≤37</td>
<td>None or &gt;37</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas</em></td>
<td>HEX</td>
<td>≤37</td>
<td>None or &gt;37</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Enterococcus and Enterobacteriacea</em></td>
<td>FAM</td>
<td>≤37</td>
<td>None or &gt;37</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>IAC</em></td>
<td>ATTO</td>
<td>≤32</td>
<td>&gt;32 or absent</td>
<td></td>
</tr>
</tbody>
</table>

If the IAC Ct is >32 or absent, the purified DNA should be diluted 10 times and a new qPCR reaction should be analyzed using this diluted DNA. A dilution of 10 times will theoretical move Ct values 3.3 higher than undiluted.
Amplification plot with X-axis showing cycle number and Y-axis showing background subtracted fluorescence. The threshold line is used to find the Ct value. Ct is the intersection between the amplification curve and the threshold line. The threshold line is calculated automatically by the instrument giving in this example a Ct value of 24.4
• *Pseudomonas*
• *Enterococcus and Enterobactericea*
• *Streptococcus sp*
• *Bacillus and Clostridium*