

DETECTION OF INHIBITORY SUBSTANCES IN MILK

Bacillus stearothermophilus Disc Assay, Charm Tablet Method

For Raw and Finished Cow and Goat Milk

[Unless otherwise stated all tolerances are ±5%]

SAMPLES

- 1. Laboratory Requirements (see CP, item 33 & 34), except _____
 - a. For Appendix N testing, see Appendix N General Requirements form, items 9 - 14 _____

APPARATUS

- 2. See Cultural Procedures, items 1-23, except _____
 - a. For Appendix N testing, see Appendix N General Requirements form, items 1-7 _____
- 3. Fixed volume or electronic pipettors: 90 µL and 500 µL (optionally 50 µL) with appropriate tips _____
- 4. Forceps, Fine Points, Stainless Steel _____
- 5. Water Bath and/or heating block, Thermostatically Controlled at 64±2C, and 82±2C _____
- 6. Incubator 64±2C _____
- 7. Vernier, Dial or Digital Calipers, metal (readable to 0.1 mm) _____
- 8. Stirring hot plate/stirring bar (optional) _____
- 9. 100 mL Class A graduate cylinder _____
- 10. 13 x 100 mm test tubes _____
- 11. 250 mL Erlenmeyer flasks _____

MATERIALS

- 12. See Cultural Procedures, items 24-32 _____
- 13. Filter Paper Discs, Blank, Unimpregnated, Non-sterile _____
(Brand: _____ Lot#: _____)
 - a. High absorbability, diameter 12.7±0.1 mm _____
- 14. Charm PM Indicator Agar _____
 - a. **Do Not Autoclave** - (see plate preparation, item 19 below) _____

15. Charm Beta-lactamase tablet or liquid concentrate (not required if beta-lactamase is not used for confirmation) _____
- a. Stored at -15C or below _____
- b. Do not use beyond expiration date _____
- Lot#: _____ Exp. date _____
- c. Reconstitute freeze dried concentrate as per manufacturer instructions _____
1. Liquid concentrate stored at -15C or below in a non-frost-free freezer or in a styrofoam box in a frost-free freezer and used within 2 weeks _____
- d. Test each lot for suitability, add beta-lactamase to 5.0 ppb positive control (item 16) and add to one (1) disc, beta-lactamase neutralizes zone produced by positive control; records maintained _____
- Zone size: _____
16. Charm 5.0 ppb Penicillin G Standard Positive Control _____
- a. Store according to label directions _____
- Lot#: _____ Exp. date _____
- b. Store and rehydrate according to label instructions _____
- c. Test for suitability each time prepared, add to one (1) disc, must produce zone 16 - 20 mm; records maintained _____
- Avg. Zone Size: _____
- d. Use rehydrated standard within 48 hours if refrigerated _____
- Date prep. _____
- e. Or, distribute sufficient amount in small containers, seal and freeze at -15C or below in non-frost-free freezer (or in a small styrofoam box, placed in center of frost-free freezer) for no more than 2 months _____
- Date prep. _____ Lab Exp. Date: _____
17. Negative Control _____
- a. Charm Zero Control Standard _____
1. Reconstitute according to label instructions _____
- Lot#: _____ Exp. date _____

2. Use rehydrated negative control within 72 hours if refrigerated _____

Date prep. _____ Lab Exp. date _____

3. Or, distribute sufficient amount in small containers, seal and freeze at -15C or below in non-frost-free freezer (or in a small styrofoam box, placed in center of frost-free freezer) for no more than 2 months _____

Date prep. _____ Lab Exp. Date: _____

b. Inhibitor Free Milk (fluid milk product with milkfat 0.00 to 3.5%, total solids < 13%) _____

1. Test for suitability, add to one (1) disc, produces no zone; records maintained _____

Zone size: _____

18. Charm Spore Tablets _____

a. Bacillus stearothermophilus tablets containing 100,000,000 (± 10 million) spores per tablet _____

Lot#: _____ Exp. date _____

ASSAY PLATE

19. Preparation of Plate _____

a. Prepare agar according to label, 3.2g/95 mL H₂O, bring agar to a boil _____

b. Promptly cool to 64 \pm 2C (Temperature Control [TC] used) _____

1. Optionally, temperature may be determined by inserting a dedicated thermometer (not used for any other purpose) directly into test agar _____

c. Add 1 spore (white) tablet to 5 mL deionized water in 13 x 100 mm test tube _____

d. Shake test tube 25 times through 1 foot arc in 7 seconds, or vortex for 10 seconds and let settle 1 minute _____

e. Repeat item d _____

f. Decant spore mixture into agar tempered to 64 \pm 2C leaving residue on bottom of tube (avoid pouring mixture down side of flask) _____

g. Mix agar well for 1.5 minutes but avoid incorporation of air bubbles, optionally use stirring bar on magnetic stir plate _____

h. Constantly mix remaining agar during preparation of plates _____

- i. Pipet 6 mL inoculated agar into plastic petri dish (15 x 100 mm, bottom plate inner diameter 86.1 - 87.0mm) _____
- j. Or, appropriate amount of agar into other size [(Dcm)² 6/8.65² = V]; Dcm = inner diameter of plate in centimeters; V = volume (mL) of agar to add in dishes, records maintained _____
- k. Plates have flat bottoms and do not buckle after agar has been added, plates observed before and after preparation for suitability _____
- l. Swirl plate gently on level surface to evenly distribute agar _____
- m. Allow agar to solidify on a level surface for 15 minutes with lid ajar _____
- n. Use within 5 days, if stored at 0-4.4C in airtight container _____

Date prep. _____ Lab Exp. date _____

TECHNIQUE

20. Laboratory Procedure, Screening _____

- a. Label bottom of plates prior to adding discs, use template as a guide to assure discs will be placed at least 10 mm from the petri dish wall and from other discs _____
- b. Each test plate may contain a maximum of 5 test sample discs plus a positive control and negative control disc (7 discs total as per template, for larger plates more discs may be placed, maintain comparable spacing) _____
- c. Mix sample/control by shaking 25 times in 7 sec. through 1 ft arc or invert retail containers 25 times or vortex for 10 seconds (allow foam to dissipate before taking sample) _____
- d. Samples/controls (maintained at 0-4.4C) must be tested within 3 min of agitation _____
- e. Procedure _____
 - 1. With tip securely fastened to the end of the pipettor and the pipettor in a vertical position, depress the plunger to the first stop or for electronic pipettors as per manufacturer _____
 - 2. With the plunger still depressed, insert tip 1 cm below surface of the sample (avoid foam) _____
 - 3. Release plunger **slowly** allowing tip to fill (quickly releasing the plunger will cause inaccurate filling and may foul pipettor) _____

4. Remove tip from sample and depress plunger to empty tip back into sample _____
 5. Press plunger to first stop and repeat 2 and 3 above _____
 6. Touch off to a dry spot on the sample container _____
 7. Using clean, dry forceps, remove a disc from its container and place the disc (using a template as a guide) on the agar surface of the inhibitor plate _____
 8. Press the disc **gently** with the forceps to insure good contact and then fill disc immediately _____
 9. With the pipettor in a vertical position and the tip about 5 mm above the center of the disc depress the plunger to the first stop in such a way as to get a rapid drop-wise release of the sample _____
 10. Sample not applied too slowly or quickly (streamed) _____
 11. Allow a second or two for the milk to absorb into the disc _____
 12. If blow out type pipettor used, press the plunger to the second stop to completely empty the tip _____
 13. **Gently** touch off the tip on an area of the disc away from where the sample was deposited _____
 14. Repeat the above until all samples have been done _____
- f. Place a positive control disc containing 5.0 ppb penicillin G and a negative control disc on each test plate using above procedure _____
1. Vary the location of positive control discs in a series of test plates, i.e. center or outside of the plate _____
- g. Invert plate(s) and incubate at $64 \pm 2^\circ\text{C}$ until well defined zones of inhibition are obtained (usually 2.5 - 3 hr) with the 5.0 ppb positive control(s), plate(s) should be yellow _____
- h. Remove plates from incubator and allow to cool on a level surface for 2 minutes (do not remove lid before plates are cooled) _____
- i. Examine positive control zone. A valid test requires a positive control zone of 16-20 mm. If zone size is < 16 or > 20 mm the test must be repeated _____
- j. Examine plate for zones of inhibition surrounding the test discs, zones of > 12.7 mm indicates presence of inhibitory substances _____

- k. Measure zones of inhibition by using calipers _____
 - 1. Use the inside diameter points (smaller points) _____
 - 2. Anchor one point in the bottom of the plate at the edge of the zone and expand calipers until the other point rests on the other edge _____
 - 3. Read calipers and report zone size to the nearest 0.1 mm _____
- l. Zones of ≤ 12.7 mm are read as no zone _____
- m. Zones > 12.7 mm must be promptly confirmed to report as positive for inhibitor or beta-lactam residue _____

21. Laboratory Procedure, Confirmation _____

- a. Inhibitor confirmation and optional beta-lactamase confirmation _____
 - 1. Heat a 0.5 mL (500 μ L) portion of each suspect sample to $82 \pm 2^\circ\text{C}$ for 2 minutes (TC required) _____
 - 2. Cool promptly in ice bath to room temperature _____
 - 3. Label bottom of plates prior to adding discs _____
 - 4. Vortex for 10 seconds, use within 3 minutes _____
 - 5. Add 90 μ L of heated samples to a disc on plate as in item 20e _____
 - 6. Use of beta-lactamase (**optional by State Regulatory Agency**) _____
 - a. Add one beta-lactamase (red) tablet to each of the heated samples and mix samples as in item 21a4 _____
 - b. Let particulates settle for 1 minute then add 90 μ L to a disc on plate (Avoid clogging pipet tip with particulates by pipetting from top of samples) _____
 - c. Or, alternatively add 50 μ L of beta-lactamase liquid concentrate (item 15c), mix samples, wait 1 minutes then add 90 μ L to a disc on plate _____
 - 7. Proceed as in items 20f-m _____
- b. Interpretation of heat treated (21a5) and optional Beta-lactamase treated samples (21a6) _____
 - 1. Inhibitor present
 - a. Zones $\geq 16\text{mm}$ of the heat treated 21a5 sample is **Positive for inhibitor**

2. Beta-lactam present (optional) _____

a. A zone around the disc containing the heat treated milk sample (21a5) but no zone around the disc containing beta-lactamase 21a6c, treated milk sample, sample is **Positive for beta-lactam** _____

b. Zones around the heat treated sample (21a5) of equal size, or < 4 mm greater, than beta-lactamase treated sample (21a6) is **Positive for inhibitor** _____

c. Zones around both the beta-lactamase treated milk sample (21a6) **and** the heat treated milk sample discs (21a5), **and**, the zone around the beta-lactamase treated milk sample disc (21a6) is ≥ 4 mm smaller than the zone around the heat treated milk sample disc (21a5) [ex. beta-lactamase = 14 mm, untreated = 18 mm], sample is **Positive for beta-lactam and inhibitor** _____

c. **Confirmation of Appendix N samples**, see Appendix N General Requirements form item 12-13, perform confirmation as in items 21a1-7 above (**use of beta-lactamase required**) and interpret as in item 21b2 above _____

22. Recording and Reporting (for Appendix N also see Appendix N General Requirements form, item 14) _____

a. Record numeric values for all measurable zone sizes for samples **and** controls (screen and confirmation), if no zone is observed record as **No Zone (NZ)** _____

b. Report presence of inhibitor only from heated milk samples _____

c. Report sample as **Positive for inhibitor** (if heat only used 21a1-5) or **Positive for beta-lactam** where demonstrated (21a6 or 21c), and zone size ≥ 16 mm _____

d. If a non-beta-lactam inhibitor is demonstrated (21a6 or 21c), report as **Positive for inhibitor** when zone size ≥ 16 mm, **report to State Regulatory Agency** _____

e. If both beta-lactam and non-beta-lactam inhibitors are demonstrated (21a1-7 or 21c), report test as **Positive for beta-lactam and inhibitor** when zone size ≥ 16 mm, **report to State Regulatory Agency** _____

f. Report numeric values for **all** measurable zone sizes for samples **and** controls _____

g. Report when zone size > 12.7 and < 16 mm as positive but Below Actionable Level _____

h. Report absence of inhibitor (no zone) as **Not Found** _____

i. If any inhibitor is present, i.e., zone > 12.7 mm, plate counts cannot be reported _____