**DEPARTMENT OF HEALTH AND HUMAN SERVICES**
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION

**MILK LABORATORY EVALUATION FORM**

| LABORATORY | | |
|---|---|---|
| LOCATION | | LAB # |
| DATE | X = DEVIATION<br>O = NOT USED | U = UNDETERMINED<br>NA = NOT APPLICABLE |

# 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, items 33 and 34), except** _____
a. For Appendix N testing, see Appendix N General Requirements form, item 9 ..... _____

### APPARATUS

**2. See Cultural Procedures, items 1 - 23, except** ..... _____
a. For Appendix N testing, see Appendix N General Requirements form, items 1-8 ..... _____
**3. Fixed volume microliter Pipettors: 90 µL and 500 µL (optionally 50 µL fixed volume pipettor)** (_____) ..... _____
**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 (see CP item 15)** ..... _____
**7. Vernier, Dial or Digital Calipers, metal (readable to 0.1 mm), small points sharp** ..... _____
**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 22 below) ..... _____
**15. Charm Beta-lactamase tablet or liquid concentrate (not required if beta-latamase 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 refrigerator or in a styrofoam box in a frost-free refrigerator and used within 2 weeks ..... _____
d. Test each lot for suitability, add beta-lactamase to 5.0 ppb positive control (item 16 or 18) and add to one (1) disc, beta-lactamase neutralizes zone produced by positive control; records maintained ..... _____
**16. Charm 5.0 ppb Penicillin G Standard** ..... _____
a. Store according to label directions ..... _____
Lot # _____ Exp. Date _____ ..... _____
b. 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 at 0 - 4.4C ..... _____
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. Phosphate Buffer** ..... _____
a. Dissolve 2 grams Potassium dibasic phosphate and 8.0 grams of monobasic potassium phosphate and make up to 1 liter. pH 6.0 ±0.05 ..... _____
**18. Na or K Penicillin G Standard (USP or Human injectable)** .... _____
a. Store according to label instructions ..... _____
Mfg. _____ Lot # _____ Exp. _____ ..... _____
b. Use a 4 or 5 place analytical balance to weigh out the penicillin G ..... _____
c. Calculate the equivalent penicillin G base by using the appropriate correction factor, potency in IU/mg ÷ potency of Pen $G^-$ (1782 IU/mg) (ex. K PenG potency = 1596 IU/mg, purity equal to 1596 ÷ 1782 = 0.895mg $PenG^-$ /mgKPenG) ..... _____
d. Make a 1 mg/mL stock solution by adding drug (100 mg $PenG^-$ ÷ item 18c) (ex. 100 ÷ 0.895 = 111.7 mg KpenG) to a 100 mL volumetric flask and making up with buffer (item 17) ..... _____
e. Make 1:100 serial dilution of the stock solution, using 100 mL volumetric flask (10 µg/mL stock) ..... _____
f. Make the final dilution in inhibitor free milk (item 19 or 20) to yield the 5.0 ppb standard (ex. 0.5 mL of item 18.e. + 999.5 mL milk = 1000mL of 5 ppb $PenG^-$) ..... _____
Date prep. _____ ..... _____
g. Test for suitability each time prepared, add to one (1) disc, must produce zone 16 - 20 mm, records maintained ..... _____
Avg. Zone Size _____ ..... _____
h. Store 5.0 ppb standard at 0 - 4.4C for no more than 2 days
i. 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 _____ ..... _____
**19. Charm Zero Control Standard** ..... _____
a. Store according to label directions ..... _____
Lot # _____ Exp. Date _____ ..... _____
b. Rehydrate according to label instructions ..... _____

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c. Test for suitability *each* time prepared, add to one (1) disc, must not produce a zone; records maintained ..... _____
Avg. Zone Size _____ ..... _____

d Use rehydrated negative control within 72 hours if refrigerated at 0 - 4.4C ..... _____
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 _____ ..... _____

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

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

**21. Charm Spore Tablets** ..... _____

a. *Bacillus stearothermophilus* tablets containing 100,000,000 (±10 million) spores per tablet ..... _____
Lot # _____ Exp. Date _____ ..... _____

## ASSAY PLATE

**22. Preparation of Plate** ..... _____

a. Prepare agar according to label, 3.2 g/95 mL $H_2O$, bring agar to a boil ..... _____

b. Promptly cool to 64 ±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 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 ±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 agar during preparation of plates ..... _____

i. Pipet 6 mL inoculated agar into plastic petri dish (15 x 100mm, 86.1 - 87.0 mm I.D.) ..... _____

j. Or, appropriate amount of agar into other size [$(Dcm)^2$ 6/ $8.65^2$ = 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. _____ ..... _____

## TECHNIQUE

**23. 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 pipet-tor in a vertical position, depress the plunger to the first stop ..... _____
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 to 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, template used
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 ±2C 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 ..... _____

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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 of the two 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 (NZ) .....

m. Zones > 12.7 mm must be promptly confirmed to report as positive for inhibitor or beta-lactam residue .....

**24. Laboratory Procedure, Confirmation** .....

a. Inhibitor confirmation

1. Heat a 0.5 mL (500 µL) portion of each suspect sample to 82 ±2C 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 µL of heated samples to a disc on plate as in item 23e .....

6. Optional 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 24a4 .....

b. Let particulates settle for 1 minute then add 90 µL to a disc on plate (Avoid clogging pipet tip with particulates by pipetting from top of samples) .....

c. Or, alternatively add 50 µL of beta-lactamase liquid concentrate (item 15c), mix samples, wait 1 minutes then add 90 µL to a disc on plate .....

7. Proceed as in items 23f - m .....

b. Interpretation of heat treated and optional Beta-lactamase treated samples .....

1. Inhibitor present .....

a. Zones ≥ 16mm of the heat treated only sample is **Positive for inhibitor** .....

2. Beta-lactam present (optional) .....

a. A zone around the disc containing the heat treated milk sample but no zone around the disc containing beta-lactamase, treated milk sample, sample is **Positive for beta-lactam** .....

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

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

c. Test for Beta-lactam (optional) .....

1. Use approved Beta-lactam screen test, if positive report as in 25c. If Not Found then **MUST** confirm for inhibitor as in 24a .....

d. **Confirmation of Appendix N samples**, see Appendix N General Requirements form item 11, perform confirmation as in items 24a1-7 above (**use of beta-lactamase required**) and interpret as in item 24b2 above .....

**25. Recording and Reporting** (for Appendix N also see Appendix N General Requirements form) .....

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) or **Positive for beta-lactam** where demonstrated (24a6 or 24c), and zone size ≥ 16 mm .....

d. If a non-beta-lactam inhibitor is demonstrated (24a6 or 24c), report as **Positive for inhibitor (other than beta-lactam)** when zone size ≥ 16 mm, **report to State regulatory agency** .....

e. If both beta-lactam and non-beta-lactam inhibitors are demonstrated (24a6 or 24c), report test as **Positive for beta-lactam and inhibitor (other than beta-lactam)** when zone size ≥ 16 mm, r**eport 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 .....