

**PHOSPHATASE TEST - CHARM[®] PASLITE[®] - ALKALINE PHOSPHATASE TEST
USING CHARM II 6000/6600 AND LUMINOMETER/LUMINATOR/NOVALUM[®]
IMS #29**

[Unless otherwise stated all tolerances are $\pm 5\%$]

SAMPLES

1. Laboratory Requirements (see Cultural Procedures [CP] items 33 & 34) _____

[See current version of M-a-98 to determine if this test method has been approved for use on the specific dairy product being tested]

a. Product Groups/Descriptions _____

1. Fluid white milks - including skim through whole fat milk _____
2. Unflavored liquid dairy products – including half and half, cream, light cream, whipping cream (products that can be accurately pipetted) _____
3. Flavored liquid dairy products (Liquid products that can be accurately pipetted, containing flavor additives and/or thickening agents including flavored milks, and etc.) _____
4. Solid/semisolid dairy products - thick dairy products not able to be pipetted, solid and/or powdered additives, including e.g.; heavy cream $\geq 36\%$ milkfat _____

APPARATUS

2. CP, items 1-32 (as necessary) _____

- a. Unless otherwise stated, “shake vigorously” refers to standard microbiological mixing, i.e., 25 times in a 1 foot arc in 7 sec or vortex for 10 sec at maximum setting (subsamples/controls in an appropriate container for vortexing) _____

3. Instrument Used: _____

- a. Charm II 6000/6600: _____
- b. Luminometer: _____
- c. Luminator: _____
- d. NovaLUM: _____

4. Incubator Block for 13 x 100 mm Test Tubes or 2 mL Microtubes _____

- a. Thermostatically-controlled at $35 \pm 1^\circ\text{C}$ _____
- b. Check temperature by electronic display or by thermometer in small well in block or by liquid immersion; maintain records _____

5. Pipettors and Pipets

- a. Fixed volume or electronic, 100 µL
- b. Calibration checked as specified in CP item 6.e; maintain records
- c. Disposable, 10 mL (ASTM) pipet with 0.1 mL graduations

6. Reagent Dispenser

- a. Fixed volume or electronic, 1.0 mL
- b. Calibration checked (CP item 6.e) with 10 mL Class A graduated cylinder; maintain records

7. Test Tubes or Microtubes and Adapter

- a. Test tubes for Charm II 6600/Charm II 6000 systems, disposable borosilicate glass 13 x 100 mm, dirt and scratch free
- b. Microtubes - for Luminometer/Luminator/NovaLUM, 2 mL screw cap
- c. Microtube adapter for Luminometer/Luminator/NovaLUM

8. 6000/6600 or Luminometer/Luminator/NovaLUM Analyzer

- a. Operating instructions available
- b. Calibrated for applicable product groups, item 1.a

9. Water Bath, Circulating, 34±1°C and 63±1°C (or 66±1°C if fat > 10%), or 13 x 100 Test Tube Dry Well Heater Blocks acceptable (Confirmation Procedure)

10. Centrifuge - Charm II Heraeus® (3,400 RPM), Minifuge, or Equivalent (1,200 - 2,000 g)

11. Handling and Storage

- a. Kit contains Reagent AP, Stopping Solution, Alkaline Phosphatase Calibrator Tablets and Positive Control

Kit: Lot #: _____ Rcd. Date: _____ Exp. Date: _____

- 1. For solid/semisolid dairy products, Diluent AP

Diluent AP: Lot #: _____ Exp. Date: _____

- b. Store reagents at 0.0-4.5°C until expiration date

- c. Stopping Solution may be stored at room temperature. If stored at room temperature, laboratory expiration date is 2 months from first date of room temperature storage. Stopping solution must be at 18-24°C at time of use

- 1. For the Charm 6600 and Luminometers without temperature probes, the stopping solution may be stored in a water bath or other means to maintain within $\pm 1^\circ\text{C}$ of the temperature used during calibration

- d. Label bottles with open and expiration dates

CONTROLS

12. Negative Control/Sample

- a. Product group. Prepare at least 50 mL of negative sample for use as a negative control, negative calibrator, and to rehydrate positive control and calibrators

- 1. Fluid white milk - heat a sample of product (highest fat content) to $95\pm 1^\circ\text{C}$ for 1 min with stirring

- 2. All flavored liquid dairy products can be tested by heating a chocolate sample (highest fat content) to $95\pm 1^\circ\text{C}$ for 1 min with stirring

- 3. All unflavored liquid dairy products can be tested by heating pasteurized light cream to $95\pm 1^\circ\text{C}$ for 1 min with stirring

- 4. Solid/semisolid dairy products - mix or knead 5 g of product (highest fat content) with 20 mL Diluent AP until homogeneous and heat to $95\pm 1^\circ\text{C}$ for 1 min with stirring. Cool on ice to 0.0-4.5°C. Centrifuge for 3 min and decant supernatant for use as Negative Control/Sample

- 5. Note, if product precipitates during negative sample preparation, e.g. sheep milk, heating sample to 63°C for 45 min is acceptable. If using 13 x 100 test tube dry well heater block at 95°C it takes 10 min to heat product to 95°C ; once at temperature, time for 1 min; (Use TC)

- b. Cool rapidly in an ice bath and hold at 0.0-4.5°C

- c. Store at 0.0-4.5°C, the Negative Control/Sample may be used for up to 48 hours

- d. Or, aliquot 1 mL quantities in small tubes (milk only), seal and freeze at -15°C or colder in a non-frost-free freezer, or place in an insulated foam container in a frost-free freezer, use within 2 months

Lab Prep. Date: _____ Lab Exp. Date: _____

13. Positive Control (for Daily Checks)

- a. Reconstitute positive control (450 mU/L) with negative control/sample, item 12, as indicated on label, or alternatively use 350 mU/L calibrator (item 14.a.2.a)
- b. Shake vigorously or vortex and let settle 10 min at $0.0-4.5^{\circ}\text{C}$ for re-suspension
 - 1. For solid/semisolid dairy products only, add 1 mL of rehydrated material 13.b with 3 mL of negative control/sample (item 12.a.4) to complete preparation of positive control
- c. Shake vigorously or vortex again and use for test
- d. Positive controls and calibrators held at 0.0 to 4.5°C may be used for 48 hours, milk controls may be frozen at -15°C or lower for up to 3 weeks; thaw in refrigerator prior to use
- e. With 6600 and C2Soft, enter either the triplicate RLU average of positive control or triplicate RLU average of 350 mU/L calibrator as the pos avg. and CP in C2Soft configuration file. Refer to C2Soft manual

CALIBRATION

14. With Each New Kit Lot # Check Calibration of Analyzer and Replace Microtube Adapter When Applicable

- a. Prepare 350 mU/L, 175 mU/L, 44 mU/L (milk only), 88 mU/L (flavored and unflavored only) calibrators using negative control/sample, item 12
 - 1. Rehydrate a calibrator tablet with 100 μL water, mix to disperse tablet, wait 1 min and mix again
 - 2. Add the specified volume of negative control/sample to each dissolved calibrator tablet to make calibrators:
 - a. Add 2.5 mL to make 350 mU/L
 - b. Add 5 mL to make 175 mU/L
 - c. Add 10 mL to make 88 mU/L (flavored and unflavored only)
 - d. Add 20 mL to make 44 mU/L (fluid white milk only)

3. Wait 10 min to rehydrate. Maintain at 0.0-4.5°C. Mix before use

b. Calibrate instrument by testing each calibration control (350, 175, 44 (or 88) mU/L) in triplicate

6600 with C2Soft Software

c. For fluid white milks, unflavored or flavored liquid dairy product on the 6600 system with C2Soft software, follow the Standard Curve Calibration procedure

1. Program has a separate assay line for each product group, fluid white milk, flavored and unflavored liquid dairy product

2. In calibrate mode, enter low concentration (44 or 88 mU/L) value, followed by 3 replicate counts

3. Enter medium concentration (175 mU/L) value, followed by 3 replicate counts

4. Enter high concentration (350 mU/L) value, followed by 3 replicate counts

5. Calibration successful will be prompted at end of the procedure

d. For solid/semisolid dairy products using the 6600 system with C2Soft, follow instructions for positive average or control point setup

1. Count 3 replicates of 350 mU/L control

2. Control point is equal to average of triplicate counts

Luminometer/Luminator/NovaLUM System

e. For fluid white milk, unflavored or flavored liquid dairy products, determine average value for each calibrator

1. Set up a separate channel and calibration for each product group, fluid white milk, flavored and flavored liquid dairy products

2. Check calibration

a. Average negative control/sample tested in triplicate. Average must be less than 5 (less than 15 for flavored dairy products)

b. Average 44 mU/L (or 88 mU/L unflavored and flavored liquid dairy products) calibrator, must be between 32-55 mU/L (45 – 110 mU/L unflavored and flavored liquid dairy products)

c. Average 175 mU/L positive control, must be 145-205 mU/L

- d. Average 350 mU/L calibrator, must be 320-400 mU/L _____
- 3. If conditions are not met, recalibrate according to Luminometer/Luminator/NovaLUM calibration instructions _____
- f. For solid/semisolid dairy products verify control point of 350 mU/kg _____
 - 1. Count 3 replicates of negative control/sample and 350 mU/kg positive control _____
 - 2. Average negative/control sample must test less than 245 mU/kg _____
 - 3. Average 350 mU/kg positive control, must test 350±105 mU/kg _____
 - 4. If conditions are not met, recalibrate according to Luminometer/Luminator/NovaLUM calibration instructions _____

DAY OF USE PERFORMANCE CHECKS

15. Each Day of Use, Test a Negative Control/Sample (item 12) and Positive Control (item 13), For at Least One Product _____

- a. Test beginning from item 16.b _____
- b. Verify negative control/sample calibration _____
 - 1. Fluid white milk test VALID or less than or equal to 5 mU/L, unflavored and flavored assay value VALID or less than or equal to 15 mU/L with Luminometer/Luminator/NovaLUM or < 44 mU/L (<88 mU/L flavored and unflavored) with 6600 and C2Soft _____
 - 2. Solid and semi-solid dairy products test VALID or less than 30% of the control point _____
- c. Verify positive control calibration _____
 - 1. Positive Control (450 mU/L) rehydrated with fluid white milk, flavored and unflavored fluid dairy products, must be 300-585 mU/L or 350 mU/L calibrator must be 247-453 mU/L _____
 - 2. Solid and semi-solid dairy products, within ± 30% of 350 mU/kg or the control point _____

TEST PROCEDURE

16. Test Procedure

[Samples kept at 0.0-4.5°C throughout testing]

a. Prepare sample

1. For fluid white milks, unflavored and flavored, mix by inverting top to bottom, then bottom to top (a complete half circle or 180 degrees) without pausing, 25 times; use within 3 min
2. For subsamples of fluid white milk, unflavored and flavored, mix by shaking 25 times in 7 sec with a 1 ft movement or vortex for 10 sec at maximum setting; use within 3 min
3. For solid/semisolid dairy products (**not including controls, items 12.b & 13**) add 1 part to 4 parts Diluent AP
 - a. Mix or knead until homogeneous
 - b. Centrifuge for 3 min
 - c. Use liquid extract in item 16.c

b. Dispense 100 µL of Reagent AP into test tubes or microtubes (do not dispense down the sides)

c. Dispense 100 µL of the prepared sample (item 16.a) or mixed controls (items 12.d & 13) just above the Reagent AP and immediately vortex

1. Use a new pipet tip for each sample, place pipet tip in sample or prepared control (no more than 1 cm), draw up and remove tip from sample/control
2. Touch off to side of container
3. Holding pipet 90° to lab bench at eye level, dry exterior of tip (if necessary) by wiping from the pipet toward the tip, be careful not to touch end of tip
4. Dispense 100 µL sample directly above surface of Reagent AP (do not dispense down side of test tube or microtube)
5. Depress plunger several times to completely expel sample
6. Mix test tubes or microtubes with a back-and-forth motion for 10 sec – or use a vortex mixer

d. Place the test tube/microtube in the 35±1°C incubator for 3 min

- e. Within 10 sec after incubation add 1 mL of room temperature (18-24°C) Stopping Solution _____
- f. Remove test tubes/microtubes from incubator, cap and shake each vigorously or vortex for 10 sec _____
- g. Place test tube/microtube into analyzer within 3 min, tubes held at room temperature (Note: stability of count may be stabilized by placing tubes/microtubes in a room temperature bath) _____

1. **6600 with C2Soft Software** _____

- a. Select appropriate assay type _____
- b. Enter ID of sample and press enter _____
- c. Load sample in analyzer and press enter _____
- d. In 5 sec RLU reading will be displayed, mU/L value will appear in results or pop-up window _____
- e. For solid/semisolid dairy products, sample RLU will be compared to control point _____

2. **Luminometer/Luminator/NovaLUM** _____

- a. Select appropriate AP calibrated channel _____
- b. Press Start or Enter _____
- c. In 5 sec mU/L reading will be displayed _____
- h. Counting of all test tubes/microtubes must be completed in 3 min _____
- i. Samples with ≥ 350 mU/L or ≥ 350 mU/kg (or for solid/semisolid dairy products, values greater than or equal to control point) of ALP activity are suspect positive and must be confirmed (item 17) _____

CONFIRMATION

17. Positive Confirmation _____

- a. Prepare lab pasteurized negative control and positive control made of the same dairy product _____
- b. Test controls to verify they are in range. If out of range, recalibrate channel and test controls to verify calibration _____
- c. Retest suspect positive sample _____

- d. Samples with ≥ 350 mU/L of ALP activity are suspect positive and must be tested for microbial, and reactivated phosphatase (items 18 & 19)

18. Microbial Phosphatase/Heat Stable Phosphatase

- a. Heat 1.0 mL of suspect sample at $63\pm 1^\circ\text{C}$ for 30 min, stirring or mixing every 10 min
 - 1. For semisolid/solid dairy products dilute 1.0 g suspect sample with 4.0 mL diluent AP, mix or knead until homogeneous
 - 2. If fat content is $> 10\%$, heat at $66\pm 1^\circ\text{C}$ for 30 min
- b. Cool sample rapidly to $0.0\text{-}4.5^\circ\text{C}$ in an ice bath
- c. Test positive and negative controls (item 17.a) following item 16
- d. Test heated sample and unheated sample (original sample) following item 16 (semisolid/solid dairy products begin at item 16.b)
- e. Interpretation
 - 1. Controls test as specified in item 15
 - 2. If heated and unheated samples have equal activity ($\pm 30\%$, mU/L or RLU) the sample is regarded **Not Found** for residual phosphatase, the activity originally measured is microbial
 - 3. If the heated sample is more than 30% below unheated sample (mU/L or RLU), the sample contains milk phosphatase activity, either residual or reactivated

19. Reactivated Phosphatase

- a. Magnesium acetate solution commercially available
- b. Or, prepared in laboratory
 - 1. Dissolve 35.4 g of Mg acetate tetra-hydrate, $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ in 25 mL deionized (DI) water, warming slightly to aid dissolution
 - 2. Pour solution into 100 mL volumetric flask, rinse original container several times and add rinses to flask
 - 3. After cooling to room temperature, make up to 100 mL (stable for 1 year at $0.0\text{-}4.5^\circ\text{C}$)

c. Procedure

1. Label separate test tubes as "Blank" and "Test"
2. Add a 5.0 mL aliquot of sample (unheated, original sample prepared as in 12.a) to each test tube
 - a. For semisolid/solid dairy products, combine 2.5 g product and 10.0 mL Diluent AP
 - b. Mix or knead until homogeneous, and add 5.0 mL to clean test tubes labeled "Blank" and "Test"
3. Add 0.1 mL DI water to the sample labeled "Blank", and 0.1 mL Mg acetate solution to the sample labeled "Test"
4. Cap tubes, mix and heat both aliquots for 1 hour at $34\pm 1^{\circ}\text{C}$
5. Remove samples from water bath and cool rapidly to $0.0\text{-}4.5^{\circ}\text{C}$ in an ice bath
6. Dilute 1 mL of sample containing Mg acetate (Test) with 5 mL (1:6 dilution) of negative control product (item 12.a) and mix; label tube as "Diluted Test"
7. Test undiluted sample containing no Mg acetate (Blank) and diluted sample containing Mg acetate (Diluted Test) for phosphatase activity following item 16 (semisolid/solid dairy products begin at item 16.b)

d. Interpretation

1. If the diluted aliquot containing Mg acetate (Diluted Test) has equal (30%) or greater phosphatase activity than the undiluted aliquot containing no Mg (Blank), the sample is regarded as **Not Found** for residual phosphatase, and the phosphatase originally measured is of reactivated origin

Diluted w/Mg (Test) \geq Undiluted (Blank) = Reactivated

2. If the diluted aliquot (Diluted Test) contains less (30% below or less) activity than the undiluted aliquot (Blank) the sample is considered **Positive** for residual phosphatase

Diluted w/Mg (Test) $<$ Undiluted (Blank) = Residual

3. A false-positive for residual phosphatase may also be obtained if a reactivatable sample has been allowed to stand at elevated temperatures (20°C) for periods of 1 hr or more before testing (SPC $<$ 20,000/mL)

RECORDING, INTERPRETATION, AND REPORTING

20. Record and Interpretation

- a. Record values
- b. Interpret
 - 1. If value obtained is < 44 mU/L for fluid white milk, < 88 mU/L for unflavored, or < 350 mU/kg for solid/semi-solid dairy products the sample is **Not Detected** (the 6600 with C2Soft software doesn't give a value but states None Found)
 - 2. If value obtained is ≥ 350 mU/L or mU/kg the sample is **actionable** (for solid/semi-solid dairy products the 6600 with C2Soft software doesn't give a value but states 'Suspect')

21. Report

- a. **Not Found** for residual phosphatase if:
 - 1. < 350 mU/L
 - 2. ≥ 350 mU/L or mU/kg but:
 - a. Meets reactivated phosphatase criteria (item 19.d.1)
 - b. Meets microbial/heat stable phosphatase criteria (item 18.e.2)
 - c. Documentation shows the product was treated in such a way that reactivated phosphatase may be present
- b. **Positive** for residual phosphatase if:
 - 1. ≥ 350 mU/L or mU/kg and:
 - a. Meets residual phosphatase criteria (item 19.d.2)
 - b. No microbial phosphatase present (item 18.e.3)
 - c. No documentation to show the product could have become reactivated