

**PHOSPHATASE TEST - CHARM® PASLITE® - ALKALINE PHOSPHATASE TEST
USING CHARM II 6000/6600 AND LUMINOMETER/LUMINATOR/NOVALUM®/NOVALUM II X®
IMS #28b**

[Unless otherwise stated all tolerances are ±5%]

SAMPLES

1. Laboratory Requirements (see Cultural Procedures [CP] items 32 & 33)

[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 milk, 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 ≥ 36% milkfat

APPARATUS

2. CP, items 1-31 (as necessary)

- a. Unless otherwise stated, “shake vigorously” refers to standard microbiological mixing, i.e., 25 times in a 1 foot movement 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: _____
- e. NovaLUM II X: _____

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

- a. Thermostatically-controlled at 35±1°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/NovaLUM II X, 2 mL screw cap
- c. Microtube adapter for Luminometer/Luminator/NovaLUM/NovaLUM II X

8. 6000/6600 or Luminometer/Luminator/NovaLUM/NovaLUM II X 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 Calibrator/Control _____

- 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 into small tubes (milk only) within 24 hours, 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°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 within 24 hours, 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 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 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/NovaLUM II X 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, unflavored 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/NovaLUM II X 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/NovaLUM II X 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/NovaLUM II X 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 \pm 30% of 350 mU/kg or the control point _____
- d. Periodic rotation of product calibrations is recommended when multiple calibrations are used _____

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 & 13**) and 1 part to 4 parts Diluent AP _____
 - a. Mix or knead until homogeneous _____

- b. Centrifuge for 3 min _____
- c. Use liquid phase in item 16.c _____
- b. Dispense 100 μ L of Reagent AP into test tubes or microtubes (do not dispense down the sides) _____
- c. Dispense 100 μ L of the prepared sample (item 16.a) or mixed controls (items 12 & 13) just above the Reagent AP and immediately mix (16.c.6) _____
 - 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 μ L 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 \pm 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/NovaLUM II X**

- 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 (-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, $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ in 25 mL deionized (DI) or MS water, warming slightly to aid dissolution _____
 2. Pour solution into 100 mL volumetric flask, rinse original container several times and add rinse to flask _____
 3. After cooling to room temperature, make up to 100 mL (stable for 1 year at 0.0-4.5°C) _____
- c. Procedure _____
 1. Label separate test tubes as "Blank" and "Test" _____
 2. Add a 5.0 mL aliquot of sample (unheated, original sample) 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 or MS 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 $34 \pm 1^\circ C$ _____
 5. Remove samples from water bath and cool rapidly to 0.0-4.5°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 _____