

**PHOSPHATASE TEST - CHARM PASLITE - ALKALINE PHOSPHATASE TEST
USING CHARM II 6000/6600 AND LUMINOMETER/LUMINATOR/NOVALUM**

[Unless otherwise stated all tolerances are ±5%]

SAMPLES

1. Laboratory Requirements (see CP, items 33 & 34) _____

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 one foot arc in seven seconds _____

3. Incubator Block for 13 x 100 mm test tubes or 2 mL microtubes _____

- a. Thermostatically-controlled at 35±1C _____
- b. Temperature checked by electronic display or by thermometer in small well in block or by liquid immersion, records maintained _____

4. Pipettors and Pipets _____

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

5. Reagent Dispenser _____

- a. Fixed volume or electronic, 1.0 mL _____
- b. Calibration checked (CP item 6e) with 10 mL ASTM graduated cylinder, records maintained _____

6. Test tubes or microtubes _____

- 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

7. 6000/6600 or Luminometer/Luminator/NovaLUM Analyzer

- a. Operating instructions available

- b. Definitions:

- 1. Fluid white milks - including skim through whole fat milk
- 2. Unflavored liquid dairy products - including half and half, buttermilk, creams (light, medium and whipping), and etc
- 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 cheese, yogurt, cottage cheese, ice cream mix, ice cream milk, whey, sour cream, and heavy cream
 - 36%, and etc

8. Water Bath, circulating, 34±1C and 63±1C (or 66±1C if fat > 10%), or 13 x 100 test tube dry well heater blocks acceptable (Confirmation procedure)

9. Centrifuge - Charm II Heraeus (3,400 RPM), minifuge, or equivalent (1,200 - 2,000 g)

10. 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. Reagents stored at 0-4.4C until expiration date _____
- c. Stopping Solution must be at 18-24C at time of use, may be stored at room temperature, expiration date marked 2 month from room temperature storage _____
- d. Bottles labeled with receive and open dates _____

CONTROLS

11. Negative Control/Sample _____

- a. Product type. 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±1C for 1 minute with stirring _____
 - 2. Flavored liquid dairy products - heat a chocolate (highest fat content) to 95±1C for 1 minute with stirring _____
 - 3. Unflavored liquid dairy products - heat pasteurized light cream to 95±1C for 1 minute 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±1C for 1 min with stirring. Cool on ice to 0-4.4C. Centrifuge for 3 minutes 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 63C for 45 minutes is acceptable. If using 13 x 100 test tube dry well heater block at 95C it takes 10 minutes to heat product to 95C for one minute, use temperature control _____
- b. Cool rapidly in an ice bath and hold at 0-4.4C _____
- c. Kept at 0-4.4C, the Negative Control/Sample may be used for up to 48 hours _____

- d. If desired, distribute 1 mL quantities in small tubes (Milk only), seal and freeze in a non-frost-free freezer, or place in a styrofoam container and place in the center of a frost-free freezer for no more than 2 months, vials labeled with preparation and expiration dates
-

12. Positive Control (for daily checks)

- a. Reconstitute positive control (450 mU/L) with Negative Control/Sample, item 11, as indicated on label, or alternatively use 350 mU/L calibrator (item 13a2a)
 - b. Shake vigorously and let settle 10 minutes at 0-4.4C for re-suspension
 - 1. For solid/semisolid dairy products only, add 1 mL of rehydrated material 12b with 3 mL of Negative Control/Sample (item 11a4) to complete preparation of positive control
 - c. Shake vigorously again and use for test
 - d. Positive controls and calibrators held at 0-4.4C may be used for 48 hours, milk controls may be frozen at -15C 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

13. With each new kit lot # check calibration of analyzer

- a. Prepare 350mU/L, 175mU/L, 44mU/L (milk only), 88 mU/L (flavored and unflavored only) calibrators using Negative Control/Sample, item 11
 - 1. Rehydrate a calibrator tablet with 100 uL water, mix to disperse tablet, wait 1 minute 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 350mU/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 minutes to rehydrate. Keep at 4.4°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/NovoLUM System

- g. 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/NovoLUM calibration instructions _____
- h. 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 245mU/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/NovoLUM calibration instructions _____

DAILY PERFORMANCE CHECKS

**14. Daily and to verify calibration, test a Negative Control/
Sample (item 11) and Positive Control (item 12), for at
least one product**

- a. Test beginning from item 15b
- 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 350mU/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

TEST PROCEDURE

15. Procedure

- a. Prepare sample
 - 1. For fluid white milks, unflavored and flavored, invert filled retail container 25 times, each inversion a full cycle down and up, shake or vortex negative control
 - 2. For solid/semisolid dairy products (**not including controls, item 11b & 12**) add 1 part to 4 parts Diluent AP
 - a. Mix or knead until homogeneous

- b. Centrifuge for 3 minutes _____
- c. Use liquid extract in item 15c _____
- b. Dispense 100 μ L of Reagent AP into test tubes or microtubes _____
- c. Dispense 100 μ L of the prepared sample (item 15a) or mixed controls (items 11d & 12) just above the Reagent AP and immediately mix _____
 - 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, expel once to pre-wet tip _____
 - 2. Draw sample or control into pipet tip, 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 seconds, use a vortex mixer if available _____
- d. Place the test tube/microtube in the 35 \pm 1C incubator for 3 minutes _____
- e. Within 10 seconds after incubation add 1 mL of room temperature (18-24C) Stopping Solution _____
- f. Remove test tubes/microtubes from incubator, cap and shake each vigorously or vortex for 10 seconds _____

g. Place test tube/microtube into analyzer within 3 minutes, 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 seconds 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 seconds mU/L reading will be displayed _____

h. Counting of all test tubes/microtubes must be completed in 3 minutes _____

i. Samples with \bullet 350 mU/L (or for solid/semisolid dairy products, values greater than or equal to control point) of ALP activity are suspect positive and must be tested for microbial, and reactivated phosphatase (items 16 & 17) _____

CONFIRMATION

16. Microbial Phosphatase _____

a. Heat 1.0 mL of suspect sample at $63 \pm 1^\circ\text{C}$ for 30 minutes, stirring or mixing every 10 minutes _____

- 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±1C for 30 minutes _____

b. Cool sample rapidly to 0-4.4C in an ice bath _____

c. Test positive and negative controls following item 15 _____

d. Test heated sample and unheated sample (original sample) following item 15 (semisolid/solid dairy products begin at item 15b) _____

e. Interpretation _____

1. Controls test as specified in item 14 _____

2. If heated and unheated sample 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 _____

17. Reactivated Phosphatase _____

a. Magnesium acetate solution commercially available _____

b. Or, prepared in laboratory _____

1. Dissolve 35.4 g of magnesium acetate tetrahydrate, $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ in 25 mL 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 0-4.4C) _____

c. Procedure _____

1. Label separate test tubes as "Blank" and "Test" _____

2. Add a 5.0 mL aliquot of sample (unheated, original sample not prepared as in 15a) 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 MS water to the sample labeled "Blank", and 0.1 mL magnesium acetate solution to the sample labeled "Test" _____
4. Cap tubes and heat both aliquots for 1 hr at 34±1C _____
5. Remove samples from water bath and cool rapidly to 0-4.4C in an ice bath _____
6. Dilute 1 mL of sample containing magnesium acetate (Test) with 5 mL (1:6 dilution) of negative control product (item 11), label tube as "Diluted Test" _____
7. Test undiluted sample containing no magnesium acetate (Blank) and diluted sample containing magnesium acetate (Diluted Test) for phosphatase activity following item 15 (semisolid/solid dairy products begin at item 15b) _____

d. Interpretation _____

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

Dil. w/Mg (Test) • Undil. (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 _____

Dil. w/Mg (Test) < Undil. (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 (20C) for periods of 1 hr or more before testing (SPC < 20,000/mL)
-

REPORT

18. Report as:

1. Residual phosphatase **Not Found (NF)**

- a. Report as < 44 mU/L (< 88 mU/L unflavored and flavored liquid dairy products or < 350 mU/kg solid/semisolid dairy products)
-

2. Residual phosphatase **Positive**

- a. Microbial and reactivatable phosphatase are not demonstrated
-

- b. Suspect positives greater than or equal ($\pm 30\%$) to 350 mU/L, category 4 or greater than the control point must be tested for microbial and reactivated phosphatase (items 16 and 17)
-

- c. Report mU/L values, mU/L range or greater than control point when equal to or greater than 44 mU/L fluid white milk, 88 mU/L unflavored and flavored liquid dairy products or 350 mU/kg semisolid/solid dairy products
-

3. Report as **Not Found** for residual phosphatase if:

- a. If microbial phosphatase present
 - b. If reactivated phosphatase present
 - c. If there is documentation to show that the product was treated such that reactivated phosphatase may be present
-