

NATIONAL STANDARD METHOD

DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY IN PASTEURISED MILK AND CREAM - FLUORIMETRIC METHOD

D 7

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division

DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY IN PASTEURISED MILK AND CREAM – FLUORIMETRIC METHOD

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AMENDMENT PROCEDURE

Controlled document reference	D 7
Controlled document title	Standard Operating Procedure for Determination of Alkaline Phosphatase Activity in Pasteurised Milk and Cream – Fluorimetric Method

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
4/ 03.05.05	1.3	1.4	1	Front page	Redesigned
			2	Status of document	Reworded
			4	Amendment page	Redesigned

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STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY IN PASTEURISED MILK AND CREAM – FLUORIMETRIC METHOD

INTRODUCTION

Scope

The procedure describes the determination of alkaline phosphatase activity in pasteurised milk, cream and liquid milk-based products using a fluorimetric method. The method is based on BS EN ISO 11816-1¹.

Background

The enzyme phosphatase in milk is destroyed by the time/temperature conditions used for pasteurisation. Detection of the enzyme indicates inadequate pasteurisation of the milk.

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1.0 PRINCIPLE

The alkaline phosphatase activity of the sample is measured by a continuous fluorimetric direct kinetic assay. In the presence of any active alkaline phosphatase enzyme in the sample a non-fluorescent aromatic monophosphoric ester substrate is hydrolysed to produce a highly fluorescent product. The amount of fluorescence produced as a result of alkaline phosphatase activity is measured at 38°C over a three minute period.

2.0 DEFINITIONS

For the purpose of this method, the following definitions apply:

Alkaline phosphatase activity

A measure of the quantity of active alkaline phosphatase present in the product, expressed as milliUnits of enzyme activity per litre of sample (mU/L).

Unit of alkaline phosphatase activity

The amount of alkaline phosphatase enzyme that catalyses the transformation of 1 µmol of substrate per minute per litre of sample.

Residual alkaline phosphatase

Alkaline phosphatase enzyme remaining in the product due to incomplete pasteurisation or contamination with raw milk.

Reactivated alkaline phosphatase

Alkaline phosphatase enzyme which was inactivated by pasteurisation but recovered its activity due to storage conditions.

Microbial phosphatase

Phosphatase enzyme produced as a result of microbial activity that is not inactivated by heating at 63°C (milk samples) or 66°C (cream samples) for 30 minutes.

3.0 SAFETY CONSIDERATIONS²⁻⁷

Normal microbiology laboratory precautions apply. The use of a dedicated discard jar is recommended. In addition the substrate buffer and reconstituted Fluorophos[®] reagent are irritants to skin, eyes and respiratory system. The use of protective gloves is advised when handling the reagents. In case of contact with eyes, flush with plenty of water and seek medical advice.

4.0 EQUIPMENT

- Fluorophos[®] fluorimeter model FLM 200 containing programmable calculator and associated printer
- Incubator block (20 well dry block) set at 38 ± 0.5°C
- Waterbath at 34 ± 1°C
- Waterbath at 63 ± 1°C or 66 ± 1°C
- Boiling waterbath
- Vortex mixer (optional)
- Reagent dispenser FLA864 to dispense 2.0 mL volumes
- Pipettor to deliver 75 µL
- Pipette tips to deliver up to 100 µL

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- Pipettes for delivering 1 mL, 5 mL and 10 mL (optional)
- Pipettors/ pipette tips for delivering 1 mL, 5 mL and 10 mL (optional)
- Disposable cuvettes (do not re-use)
- Print rolls
- Test tubes or other glass containers (optional)
- Parafilm or other suitable laboratory grade film (optional)

5.0 REAGENTS

Fluorophos[®] ALP test kit KFLA 225

This contains:

Fluorophos[®] substrate (a water-soluble, non-fluorescent aromatic monophosphoric ester)

Substrate buffer pH 10.0 (diethanolamine [DEA] buffer)

Disposable cuvettes

Store the reagents at 2 – 6°C. The unreconstituted reagents are stable for 2 years from production at this temperature. Store the cuvettes at room temperature.

For use, pour the contents of the substrate buffer bottle into one Fluorophos substrate bottle. Mix by gentle inversion for 3 minutes to dissolve all the substrate and allow to stand for 15 minutes before use. After reconstitution attach the reagent dispenser to the bottle. Record the date of reconstitution on the bottle. The reconstituted substrate is stable for 60 days at 2 – 6°C. Protect the reagent from light. Do not keep the reagent at 38°C for more than 2 hours.

Working calibrators - Fluorophos[®] KFLA 250 Calibrator kit

This contains three solutions containing variable amounts of Fluoroyellow in DEA buffer:

Solution A: contains 0 µmol/L of fluoroyellow

Solution B: contains 17.24×10^{-3} µmol/L of fluoroyellow

Solution C: contains 34.48×10^{-3} µmol/L of fluoroyellow

Calibrator solutions are stable for 18 months when stored at $4 \pm 2^\circ\text{C}$.

Magnesium acetate solution

(40.1 mg of Mg⁺⁺/mL)

Dissolve 35.4 g of Mg(C₂H₃O₂)₂.4H₂O in about 50 mL of distilled water, with warming; then bring to 100 mL with additional distilled water. This solution is stable for 1 year at 3 – 5°C.

Fluorophos[®] Phosphacheck[™] ALP Pasteurisation Controls KFLA 260 (optional)

Contains negative, positive and Phosphacheck-N controls. Reconstitute the controls by adding 3.0 mL of deionised water to each vial. Replace the stopper, mix gently for one minute, then stand for 15 minutes before use. Do not shake controls or allow them to foam. The positive control is typically around 400 ± 40 mU/L but varies from batch to batch; check product insert with each kit for value. The negative control value is <10 mU/L. The Phosphacheck-N control can be used as an interfering substance control or to verify the calibration status of the milk product channels (not butter or cheese channels).

Unopened, unreconstituted controls are stable for one year from production when refrigerated. Once reconstituted, these are stable for 3 days when refrigerated.

Negative control (optional)

This can be prepared from test milk heated to 95°C, held for 1 minute, then cooled rapidly.

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Positive control (optional)

A positive control with phosphatase activity at or near the failure level can be produced by adding 0.1 mL of mixed-herd raw cows' milk to 100 mL of milk previously heated to 95°C for 1 minute then cooled.

Daily Instrument Control kit FLA 280 (optional)

This contains three bottles of a reference solution used for monitoring the stability of the fluorimeter. Mix gently before use. Unopened, the reagent is stable for 18 months when refrigerated.

6.0 METHOD

6.1 Preparation of samples

Phosphatase activity should be measured within 48 hours of sampling. Keep the sample below 5°C but above 0°C during storage. It is not necessary for the sample to be prewarmed before testing. Mix all samples thoroughly before sampling. To aid pipetting of thick cream samples, the cream may be warmed briefly (usually <1 min) at 37°C until liquid.

Note: If testing a batch of samples, it is advisable to remove a sub-sample to a separate container after thorough mixing and to replace the original sample in the refrigerator immediately. If a significant level of phosphatase is then detected in the subsample, a further aliquot of the refrigerated sample can then be tested for reactivation and other tests. Extended storage at ambient temperature during testing may result in reactivation.

6.2 Calibration

Establish a calibration curve for each type of product to be tested. Calibration curves are stable and need to be run when a new lot/batch of calibrators is put into use. Calibration should also be performed when the fluorimeter is initially installed, whenever service procedures may have affected the stored calibration parameters, and when assayed control values indicate unacceptable results.

There are 21 channels each assigned to a different product type. Prepare a calibration curve for each product type using the appropriate assigned channel. Cream, light cream and heavy cream are assigned to channels 10, 11 and 12 respectively. These are equivalent to whipping cream (product 10), single cream (product 11) and double cream (product 12). Product 15 - 20 are assigned as Products A - F on the display; these can be assigned to products of the user's choice e.g: goats' milk. See Appendix.

Gently invert each bottle of calibrator solution before use. Label two cuvettes for each calibrator. Dispense 2.0 mL of each calibrator in duplicate into the appropriately labelled cuvettes. Place the cuvettes in the heating block and pre-warm to 38°C for 10 minutes. Use the pipettor and a new tip to dispense 75 µL of well mixed test sample to each of the cuvettes. Use the reverse pipetting technique and wipe the outside of the pipette tip after drawing up the sample. Dispense the sample into the cuvette by depressing the plunger again to the first stop only, avoiding contact with the liquid contents but touching the tip against the side of the cuvette. Mix using the vortex mixer. Replace the cuvettes in the heating block. Complete the calibration within 10 minutes of adding the sample to the calibrators.

Note: When using the reverse pipetting technique, fully depress the pipettor plunger when drawing up the sample then depress the plunger to the first stop only when discharging the sample.

Press the CALIB switchpad. The upper corner of the CALIB pad will illuminate and remain lit until calibration is complete. The display will identify the last product tested. Select the product type to be calibrated by pressing the < or > switchpad (or use the numbered switchpads) as necessary to display the product type to be calibrated.

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When the product type is being displayed, select it by pressing the ENTER pad. If the fluorimeter has calibration values in its memory from a previous calibration on the selected product, the instrument will now print the stored values. The display should now show "INSERT A STD: [START]".

Set the fluorimeter to zero fluorescence using a cuvette containing sample and calibrator A. Open the cuvette holder door of the fluorimeter and place the cuvette in the holder. Close the door. Press the START pad. After 30 seconds, the fluorimeter will make a final determination, set the display to approximately 200 FLU, then prompt the operator to proceed with the second sample of calibrator A by again displaying "INSERT A STD; [START]". Remove the cuvette from the cuvette holder. Place the second cuvette containing sample and calibrator A in the cuvette holder and shut the door. Press START. Repeat the process with the B and C calibrators, following the prompts on the fluorimeter's display.

When all the calibrations have been performed, the display will show "CALIBRATION COMPLETE", the CALIB pad illumination will turn off and the printer will print out the new calibration data. These data are stored in the fluorimeter and are applied each time the appropriate product type is tested until recalibration is performed. If the calibration is not accepted by the fluorimeter prepare new calibrators with the selected product and repeat the entire calibration procedure.

6.3 Determination of alkaline phosphatase (ALP) activity

Ensure that the dispenser is flushed through and completely charged by depressing the plunger two or three times. Then dispense 2.0 mL of Fluorophos substrate into a labelled, new cuvette. Place the cuvette in the incubator block and pre-warm to 38°C for 10 minutes. Use the pipettor with a new pipette tip to draw up 75 µL of sample using the reverse pipetting technique. Wipe the outside of the pipette tip. Discharge the sample into the cuvette containing the pre-warmed substrate, touching the side of the cuvette just above the reagent; do not touch the reagent. Mix using the vortex mixer, or cover with parafilm and gently invert the cuvette to mix. Place the cuvette in the cuvette holder of the fluorimeter then shut the door. Press the TEST switchpad, "ALP Dairy" appears, then press ENTER. Select the product type by using the < or > pads or using the numbered switchpads, then press ENTER. Key in the sample identification number using the numbered pads then press ENTER. Then press the START pad to begin the test.

Note: The test must be started within 20 seconds after addition of the product to the working substrate.

The display will count down 60 seconds while the substrate and sample are being warmed to 38°C. After 60 seconds, the fluorimeter will begin to measure and display the fluorescence in the sample in fluorescence units (FLU). The display will start at around 200 FLU and slowly increase over the next 2 minutes. At the end of three minutes, the fluorimeter will automatically perform the necessary calculations and display the sample identification number, the average increase in fluorescence and the ALP activity in mU/L. This information will then be printed. Initial the printout to identify the operator.

If the level of ALP activity exceeds 100 mU/L, determine the origin of the phosphatase by performing a differential test for reactivation and a test for the presence of microbial phosphatase.

Also perform a test for the presence of interfering substances.

6.4 Manual calculation of results

Occasionally the printout indicates an unstable reading. If this happens again when the test is repeated the phosphatase activity may be calculated manually.

Note: If unstable readings are repeatedly obtained, perform an A/D test to check the performance of the machine and the suitability of the substrate.

If results are to be calculated manually, use the following formula:

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$$\text{ALP (mU/L)} = \frac{F_{av} \cdot C_B}{\overline{R}_c \cdot V_s} \times f$$

where

F_{av} is the average amount of fluorescence per minute

(Fluorescence at beginning of minute 2 + Fluorescence at end of minute 3 ÷ 2)

R_c is the calibration ratio for the product type

C_B is the concentration of fluoroyellow in calibrator B in $\mu\text{moles per 2 mL of calibrator}$ (3.448×10^{-5})

V_s is the volume of sample in mL (0.075 mL)

f is the factor to convert from U/mL to mU/L (1×10^6)

This equates to $F_{av} \times 459.7$

$$\overline{R}_c$$

6.5 Detection of microbial phosphatase

Pipette 1 - 5 mL of sample into a labelled bijou or test tube, then replace cap. Place the container in the waterbath set at 63°C (66°C if the fat content exceeds 10%; this will be applicable to all cream samples). Ensure that at least two-thirds of the container is below the level of the water, or the water level is at least 4 cm above the sample level. Heat for 30 minutes, gently mixing the sample every 10 minutes. Remove the container from the waterbath and cool for at least 5 minutes in cold water. Retest the sample for phosphatase activity as described above. If the phosphatase level of this heated sample still exceeds 100 mU/L the reading is due to the presence of microbial phosphatase and the original sample was properly pasteurised.

6.6 Differential test for reactivation⁸

Place 10 mL of the sample in a suitable glass container and heat in a boiling waterbath for 1 minute after the test product temperature reaches 95°C. Cool rapidly for 5 minutes.

Place 5.0 mL of unheated sample in each of two test tubes. Label one tube "blank" and add 0.1 mL of deionised water. Label the second tube "test" and add 0.1 mL of magnesium acetate solution. Cap both tubes and mix well using the vortex mixer. Incubate at 34°C for 1 hour. Remove the test tubes and cool rapidly for 5 minutes.

Perform a phosphatase test on the "blank" sample as described above.

Add 1.0 mL of the "test" sample to 5.0 mL of heated, cooled test product (1 + 5 dilution). Perform a phosphatase test on this "diluted test" sample.

If the phosphatase activity of the "diluted test" sample (1 + 5 dilution) containing magnesium ions has equal or greater activity than the undiluted sample containing no magnesium ions (the "blank" sample), the original product is considered negative for residual alkaline phosphatase activity, indicating that the phosphatase level originally measured is of reactivated origin. If the "diluted test" sample contains less phosphatase activity than the undiluted sample, it is considered positive for residual mammalian phosphatase (provided that the initial alkaline phosphatase test was positive).

Note: A false positive test for residual phosphatase may be obtained if a reactivatable sample is stood at elevated temperature (21 - 24°C) for ≥ 2 hours.

6.7 Control tests

6.7.1 A/D Test

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The A/D test is used to check the proper functioning of the equipment by measuring the accuracy of the A/D conversion channel and monitoring the A/D channel for drift over time or temperature. This should be performed daily before testing commences.

Dispense 2.0 mL of ALP Daily Instrument Control (or calibrator C) into a new, labelled cuvette. Place cuvette in the incubator block and pre-warm to 38°C for 5 minutes.

Access the A/D test through the SETUP menu. Press SETUP key, then select menu item A/D. Test by pressing < or >. With nothing in the cuvette holder, press START. Numbers will appear on the display screen; allow reading to stabilize. The display should read 302 ± 4 .

Insert cuvette of Daily Instrument Control reagent into the cuvette holder, close lid. Wait until the display is stable. A reading of 602 ± 12 should be obtained. Record the value. If outside this range use the small screwdriver supplied with the machine to turn the potentiometer screw on the left hand side of the machine. Turn the screw slowly clockwise or anticlockwise as necessary until the display reads 602. Press STOP twice to end the test.

The A/D test can also be used to test the suitability of ready to use substrate. Freshly made substrate alone in A/D mode usually gives a reading of around 500; this increases over time and the substrate should not be used if a reading greater than 1200 is obtained.

6.7.2 Positive and negative controls

Positive and negative controls are available in the Phosphacheck™ ALP Pasteurisation Control kit (KFLA 260). The positive and negative controls are used to verify the test procedure and the reliability of the reagents. Include a negative control with each batch of samples. Perform a positive control at least once a month and every time a sample gives a value greater than 100 mU/L. Assign a separate channel for testing the positive and negative controls or use channel 13 (assigned to dry milk; dried milk is used as the base ingredient of the controls). First calibrate the channel using the negative control. Then test the negative and positive controls by substituting them for the product sample in the ALP test. The instrument display and printout should record a reading of <10 mU/L of activity for the negative control. The positive control should give a reading within the range assigned for the reagent batch and printed on the product insert, typically 408 ± 40 mU/L.

6.7.3 Phosphacheck-N control

The Phosphacheck-N control may be used to verify the calibration status of the milk product channels (not butter or cheese channels). Perform this test daily on each channel in use. For most milk products, substitution of the Phosphacheck-N control for the product in the alkaline phosphatase activity test should give a result of <40 mU/L. For chocolate milk, the result should be below 70 mU/L. If the result is above these values the channel should be re-calibrated. The reagent may also be used in the Interfering Substances Control test.

6.7.4 Interfering substances control test

This test should be performed if a sample test result of >100 mU/L is obtained. Add 75 µL of sample to 2.0 mL of pre-warmed Phosphacheck N (or Calibrator A; contains 0 mU/L). Mix with vortex mixer. Run this sample through the test procedure. A reading of <10 mU/L activity should be obtained using either control. If the reading is higher than this there is evidence of the presence of interfering substrates.

7.0 REPORTING OF RESULTS

Report the level of phosphatase activity as a numerical value of mU/Litre of sample.

If the level equals or exceeds 100 mU/L, perform further tests to ascertain the origin of the phosphatase. Repeat the alkaline phosphatase test to confirm the original result. If this is confirmed, report the original value obtained. Also perform the interfering substances test, the microbial phosphatase test and the differential test for reactivation. Then report in the following way:

The level of phosphatase detected is due to residual bovine (caprine/ovine) phosphatase

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or: The level of phosphatase detected is due to microbial phosphatase
or: The level of phosphatase detected is due to reactivation

Note: Both microbial phosphatase and reactivation can occur in the same sample.

8.0 INTERPRETATION

If the phosphatase level is below 100 mU/L, the sample is deemed satisfactory.

If residual alkaline phosphatase is detected at a level between 100 and 500 mU/L, there is evidence of inadequate pasteurisation and/or post-processing contamination with raw milk. Although this satisfies the legal requirement, it represents a public health hazard and further investigation is needed. The comment should be:

Level satisfies statutory requirement; the low level of phosphatase detected is due to presence of bovine (ovine/caprine) phosphatase. Further sampling is indicated.

If the phosphatase level exceeds 500 mU/L, the comment should be:

The phosphatase level exceeds the equivalent of the standard specified in the Dairy Product (Hygiene) Regulations 1995⁹; indicates inadequate processing or contamination with unpasteurised milk.

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Appendix: Flowcharts showing the process for the determination of alkaline phosphatase activity activity in pasteurised milk and cream – fluorimetric method

PRODUCT CALIBRATION

Invert bottles of calibrator solution



Dispense 2 mL of Calibrator A into two cuvettes labelled A
Dispense 2 mL of Calibrator B into two cuvettes labelled B
Dispense 2 mL of Calibrator C into two cuvettes labelled C



Place cuvettes in 38°C heating block for 10 minutes



Add 75 µL of well mixed sample to each cuvette, vortex to mix.
Replace cuvettes in heating block.



Press CALIB pad, key in product number, press ENTER



Insert first cuvette labelled A when prompted by display



Repeat with second cuvette labelled A when prompted by display



Repeat with cuvettes labelled B



Repeat with cuvettes labelled C



Complete calibration test within 10 minutes of adding sample
Calibration data will be printed and stored

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CONTROL TESTS

A/D Test

Pre-warm 2 mL of Daily Instrument Control



With cuvette holder empty and lid shut, press SETUP, select A/D, press START



Reading should be 302 ± 4



Place cuvette of Daily Instrument Control in holder, close lid



Reading should be 602 ± 12

Positive, Negative and Phosphacheck-N Controls

Add 75 μ L of each control to 2 mL of pre-warmed substrate; perform ALP test



Reading for positive control should be within assigned value (typically 408 ± 40)

Reading for negative control should be <10

Reading for Phosphacheck-N control should be <40

Frequency of Control tests

Daily

AD test, substrate control test, negative control test, Phosphacheck-N test on each channel in use

If result is ≥ 100 mU/L: Interfering substances control test, Positive control test

Monthly (minimum):

Positive control test

ALKALINE PHOSPHATASE TEST FOR MILK AND CREAM

Warm 2 mL substrate in cuvette in heating block (38°C) for 10 minutes



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Add 75 µL well mixed sample to substrate and vortex to mix



Place cuvette in fluorimeter and close lid



Press TEST, "ALP Dairy" appears, press ENTER



Key in product type code and press ENTER



Key in Lab. No., press START (within 20 sec of adding sample)



Result is printed after 3 minutes; initial printout



If level exceeds 100 mU/L,
Repeat Alkaline Phosphatase Test
Perform Microbial Phosphatase Test
Perform Differential Test for Reactivation
Test for Interfering Substances

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ADDITIONAL TESTS FOR SAMPLES WITH LEVELS >100 mU/L

MICROBIAL PHOSPHATASE TEST

Heat 5 mL of milk sample in 63°C waterbath for 30 min
Heat 5 mL of cream sample in 66°C waterbath for 30 min



Mix gently every 10 minutes



Cool rapidly



Test 75 µL for phosphatase activity



If no significant reduction in level, original result due to microbial phosphatase

INTERFERING SUBSTANCES TEST

Warm 2 mL of Phosphacheck-N or Calibrator A in cuvette in heating block (38°C)
for 5 minutes



Add 75 µL of well mixed sample and mix with vortex



Perform phosphatase test



If value >10 mU/L, interfering substances are present

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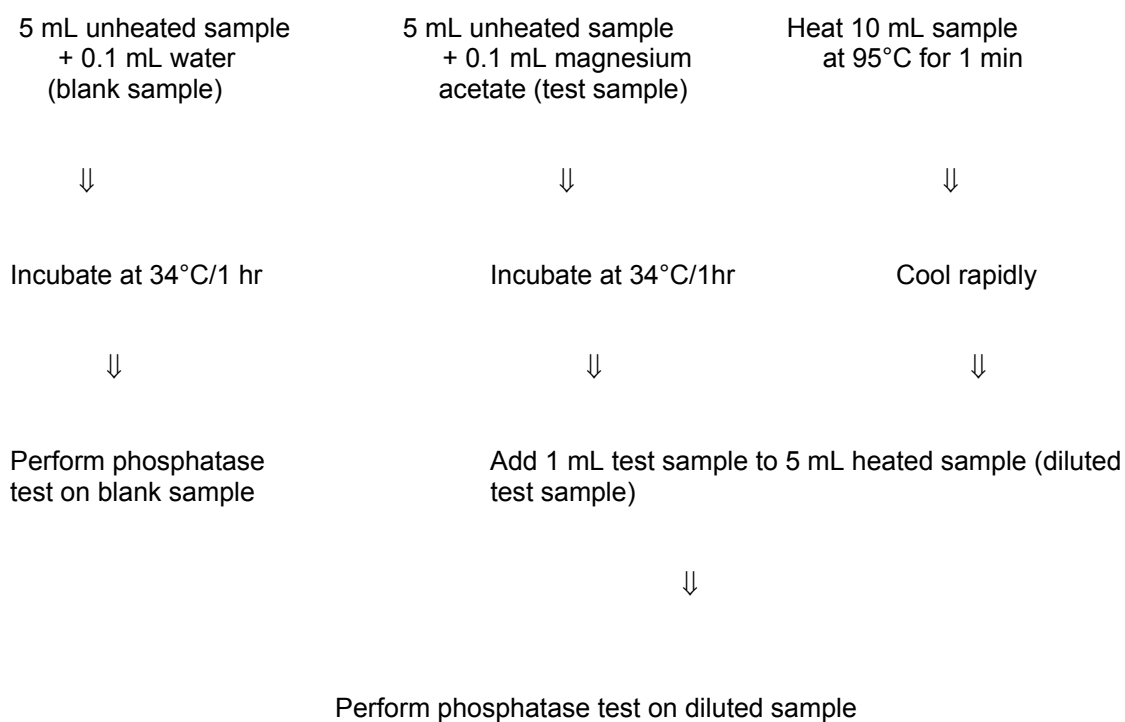
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DIFFERENTIAL TEST FOR REACTIVATION



If phosphatase level of diluted test sample \geq blank sample, reactivation has been demonstrated.

If phosphatase level of diluted test sample $<$ blank sample, original result is due to mammalian phosphatase.

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Table 1: PRODUCT LIST

1. Whole milk
2. Low fat milk 1% (semi-skimmed)
3. Low fat milk 2%
4. Skimmed milk
5. Chocolate milk
6. Cheese
7. Butter
8. Buttermilk
9. UHT milk
10. Cream (whipping)
11. Light cream (single)
12. Heavy cream (double)
13. Dry milk
14. Sour cream
15. Product A – Clotted cream
16. Product B – Goats' milk
17. Product C – Phosphacheck positive and negative control
18. Product D – Channel Island milk
19. Product E – Sheep's milk
20. Product F – unassigned
21. Drug screen

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