
**Milk and milk-based drinks —
Determination of alkaline phosphatase
activity — Enzymatic photo-activated
system (EPAS) method**

*Lait et boissons à base de lait — Détermination de l'activité de la
phosphatase alcaline — Méthode par un système de photoactivation
enzymatique*

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Contents

Page

Foreword.....	iv
1 Scope	1
2 Terms and definitions.....	1
3 Principle.....	1
4 Reagents.....	2
5 Apparatus	3
6 Sampling.....	3
7 Preparations	4
7.1 Test samples	4
7.2 Alkaline phosphatase-free milk.....	4
8 Procedure (see Annex A)	4
8.1 Calibration	4
8.2 Calibration adjustment.....	6
8.3 Control tests and calibration check.....	9
8.4 Determination.....	10
8.5 Heat-resistant microbial alkaline phosphatase control.....	10
9 Calculation and expression of results.....	10
9.1 General.....	10
9.2 Manual calculation.....	10
9.3 Expression of results	11
10 Precision.....	11
10.1 Interlaboratory test	11
10.2 Repeatability.....	11
10.3 Reproducibility.....	12
11 Test report	12
Annex A (informative) Procedure flowchart	13
Annex B (informative) Results of interlaboratory trials.....	14
Bibliography	16

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 22160|IDF 209 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

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Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

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ISO 22160|IDF 209 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Heat treatment*, of the Standing Committee on *Minor components & characterization of physical properties*, under the aegis of its project leader, Mr R. Salter (US).

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Milk and milk-based drinks — Determination of alkaline phosphatase activity — Enzymatic photo-activated system (EPAS) method

1 Scope

This International Standard specifies a method for the determination of the alkaline phosphatase activity in pasteurized whole milk, semi-skimmed milk, skimmed milk, cream and flavoured milks using a chemiluminescent (EPAS) method.

The method is applicable to milk and milk-based drinks from cows, sheep, buffalo and goats.

The method is also suitable for any liquid-based sample if diluted in such a way that the diluted alkaline phosphatase activity has less than 7 000 milliunits per litre.

NOTE There has been a successful collaborative trial with cow, sheep, buffalo, and goat whole milk, as well as skimmed cow milk (< 0,5 % fat), 20 % fat cream and 2 % fat chocolate milk (all mass fractions).

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1

alkaline phosphatase activity

ALP

activity of the alkaline phosphatase present in the product, determined according to the procedure described in this International Standard

NOTE The alkaline phosphatase activity is expressed as milliunits of enzyme activity per litre (mU/l) [4], [5].

2.2

unit of alkaline phosphatase activity

amount of alkaline phosphatase enzyme that catalyses the transformation of 1 μmol of stable aromatic substrate per minute

3 Principle

The alkaline phosphatase activity is measured by photo-activation of the hydrolysed product followed by an instrumental measurement of photo-activation. In the presence of alkaline phosphatase, a stable aromatic dioxetane-phosphate substrate is hydrolysed at $35\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ to produce a photo-activated (chemiluminescent) product. The photo-activation of the product is amplified by a macromolecular enhancing component. The hydrolysis reaction is stopped after a specified incubation time (3 min). The amount of chemiluminescent product thus produced is measured and converted to enzyme units by a luminometer. Luminometer calibration is based on calibration using tablets with known enzyme activity.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

4.1 Non-chemiluminescent dioxetane ester substrate [0,2 mol/l 3-(2'-spiroadamantanane)-4-methoxy-4-(3"-phosphate phenyl-1,2 dioxetane disodium salt in DEAE buffer with 1 % fluorescein)], is commercially available [e.g. as Charm reagent AP[®] 1) liquid].

It is recommended to store the substrate at between 0 °C and 7 °C. If stored in amber plastic vials and at 4 °C, the substrate remains stable for 6 months. If stored at 30 °C, the substrate is stable for 24 h only.

When using the substrate in the assay, keep it at between 0 °C and 7 °C or on ice.

4.2 Stopping solution

Prepare the stopping solution by mixing the same amount of 0,15 mol/l 2-amino-2-methyl-1-propanol and 0,02 % benzalkonium chloride, pH 10,7. The stopping solution shall be at room temperature (18 °C to 24 °C) prior to use. For assay consistency when not using a thermoprobe, record and maintain the temperature to within 0,5 °C of the solution temperature used at calibration.

NOTE The stopping solution is used to stop enzymatic hydrolysis of the dioxetane ester substrate (4.1).

Commercially supplied stopping solution has a shelf life of 1 year when kept at 4 °C, or of 2 months when kept at room temperature. For daily use, room temperature storage is recommended.

4.3 Working calibrators, for example, calibration tablets (dried raw milk with measured phosphatase content in tablet form to rehydrate in milk) with a phosphatase activity of 875 µU/l ± 26 µU/l. Rehydrate the tablets in three different volumes of milk-based drink presenting no phosphatase activity or negative test sample (7.2) to create a standard calibration curve.

Commercially supplied calibration tablets may be stored at 4 °C for 2 years.

4.3.1 Fluid white milk calibrators

In each of three 50 ml test tubes (5.9), marked A₁, B₁ and C₁, respectively, dissolve one calibrator tablet in 100 µl of distilled water.

Add 20 ml to tube A₁, 5 ml to tube B₁ and 2,5 ml to tube C₁ of fluid white milk products (presenting no phosphatase activity) or negative test sample (7.2). This makes calibration standards A₁, B₁ and C₁ with a phosphatase activity of A₁ = 44 mU/l, B₁ = 175 mU/l and C₁ = 350 mU/l, respectively. Cap the tubes and shake their contents vigorously. Allow the tube contents to rehydrate under refrigeration for 10 min. Mix well before use.

4.3.2 Cream and flavoured-milk calibrators

In each of three 50 ml test tubes (5.9), marked A₂, B₂ and C₂, respectively, dissolve one calibrator tablet in 100 µl of distilled water.

Add 10 ml to tube A₂, 5 ml to tube B₂ and 2,5 ml to tube C₂ of cream or flavoured-milk products (presenting no phosphatase activity) or negative test sample (7.2), making calibration standards A₂, B₂, C₂ with phosphatase activities of A₂ = 88 mU/l, B₂ = 175 mU/l and C₂ = 350 mU/l, respectively. Cap the tubes and shake their contents vigorously. Allow the tube contents to rehydrate under refrigeration for 10 min. Mix well before use.

1) The reagents specified in Clause 4 and the apparatus specified in 5.1 are available from Charm Sciences Inc., 659 Andover St., Lawrence, MA 01843, USA. These are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of these products. Equivalent products may be used if they can be shown to produce the same results.

4.4 Positive control

As positive control, use freeze-dried alkaline phosphatase in a 15 ml amber bottle. Rehydrate the positive control with 10 ml of a representative dairy drink presenting no phosphatase activity, or with a prepared negative test sample (7.2). Rehydrated positive control contains 450 mU/l of phosphatase enzyme.

Allow the control to stand for 10 min to rehydrate. Vigorously shake before use.

The rehydrated positive control is stable when stored at between 0 °C and 7 °C for 48 h. Positive control rehydrated with fluid milk is stable frozen at or below –15 °C for 2 months. Thaw the positive control in water at room temperature. Vigorously shake the control to homogenize before use. Do not re-freeze.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Luminometer, capable of operating at a wavelength of 540 nm, with linear outputs being converted by internal software into enzyme activity [e.g. Charm Luminometer® models NovaLum, Luminator K or T¹⁾]. A vial adapter is needed with NovaLum and Lum-T model. NovaLum and Lum-T should be used in the propped up position. A temperature probe supported by NovaLum should be used to measure the stopping solution (4.2) temperature.

Measurements should be optimized according to the manufacturer's recommendations for the apparatus used.

5.2 Mini-vials, disposable, made of non-luminescent plastic, with caps, and of capacity 2 ml.

5.3 Fixed volume pipette, of capacity 100 µl.

5.4 Fixed-volume dispenser, capable of dispensing 1,0 ml. Check that the volume of water dispensed is accurate to 1,00 g ± 0,05 g before use.

5.5 One-mark volumetric flasks, of capacity 100 ml.

5.6 Analytical balance, capable of weighing to the nearest 1 mg.

5.7 Incubator block or dry well bath, capable of operating at 35 °C ± 1 °C and 63 °C ± 0,2 °C with wells of mini-vial dimensions.

5.8 Water bath, adjustable, capable of operating at 63 °C ± 0,2 °C and 95 °C ± 2 °C.

5.9 Test tubes, of capacity 50 ml, of diameter 13 mm and length 100 mm, with leakproof caps.

5.10 Printer, with connection cables for printout of results.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50.

7 Preparations

7.1 Test samples

7.1.1 General

Carefully mix all test samples prior to use. The ambient temperature of the room should be between 18 °C and 24 °C.

Samples should be tested at refrigeration temperature (0 to 7) °C or on ice during assay.

7.1.2 Pasteurized samples

Use the test samples as obtained in amounts as required.

7.1.3 Raw milk

Pipette 1 ml (or an appropriate amount such that the activity after dilution is less than 7 000 mU/l) of test sample into a 100 ml one-mark volumetric flask (5.5). Dilute to the mark with alkaline phosphatase-free milk (7.2). Mix carefully.

7.1.4 Flavoured-milk products and cream products

Use the test samples as obtained in amounts as required. Test samples of viscous products may require a pipetting accuracy determination using mass (100 µl = 100 mg). Wipe the pipette tips after drawing the sample. Possibly, modification of the pipette tip (cutting wider opening) might be required for accurate dispensing.

7.2 Alkaline phosphatase-free milk

Prepare alkaline phosphatase-free milk (negative test sample) by heating 35 ml or necessary volume of a test portion (7.1.2, 7.1.3 or 7.1.4) in a tube in a water bath (5.8) set at 95 °C. Allow the test portion to reach 95 °C and keep it at this temperature for 1 min. Then cool rapidly.

The negative sample is used as the 0 (zero) calibrator and shall have a mean value of less than 5 mU/l (or less than 15 mU/l with flavoured-milk products and cream products) in the appropriately calibrated luminometer channel. If stored at 4 °C, the negative sample may be kept for 48 h.

Fluid white milks may be kept for up to 6 months if stored frozen at or below -15 °C. Thaw the negative sample in water at room temperature. Vigorously shake the sample to make it homogeneous before use. Do not re-freeze.

It should be noted that some milk products, such as sheep milk, will precipitate and separate when the negative test sample is prepared at 95 °C for 1 min. In this case, a lower temperature for a longer time is required, e.g. 63 °C for 30 min.

8 Procedure (see Annex A)

8.1 Calibration

8.1.1 Establish a calibration curve for each type of product to be tested. Begin by setting the luminometer background (B_g) to 100 and the correction (C_r) to 100.

Consult the luminometer manual for instructions on making adjustments. Calibration curves are stable and need to be run when new batch lots/lot numbers of the dioxetane ester substrate (4.1) and stopping buffer (4.2) are used. A flowchart for the calibration is given in Annex A.

NOTE The calibration mode on some luminometers automatically guides users through steps 8.1.1 to 8.2.2. (The calibration menu is item 8 of the main menu.) Select alkaline phosphatase calibration. Select channel to calibrate. Select calibrate. Select test portion to calibrate, for example milk, cream or chocolate (flavoured-milk product) or other. The luminometer prompts the user for the next steps.

8.1.2 Limit calibration assays to three tubes per assay. Use the fixed-volume pipette (5.3), provided with a clean tip, to add 100 µl of the dioxetane ester substrate (4.1) to the bottom of three mini-vials (5.2).

8.1.3 For the test portions obtained in 7.1.2 to 7.1.4, add, with the fixed-volume pipette (5.3) provided with a clean tip, 100 µl of negative test sample (7.2) to each vial. Deliver the contents of the sample to the bottom of the tube to ensure all of the sample contacts the substrate. Mix the contents of the vials in the vial rack with a back and forth motion for about 10 times in 5 s.

8.1.4 Place the mini-vials (8.1.3) in the incubator block (5.7) set at 35 °C for 3 min. At the end of incubation, add, with the fixed-volume dispenser (5.4), within 15 s, 1,0 ml of stopping solution (4.2) to the contents of all mini-vials.

8.1.5 Remove the mini-vials from the incubator. Cap each vial and shake each vigorously for 5 s. If necessary unscrew the cap and attach the vial to the luminometer adapter. Insert the vial into the luminometer. Read and record the readings of the luminometer (5.1) at count completion (beep). Repeat with each vial. Do not touch the liquid solution with the adapter to avoid contaminating the next vial. If the solution does contact the vial, rinse it with water and dry before re-use. Calculate the mean negative value (N). Repeat the determination specified in 8.1.2 to 8.1.5 if any one value varies by more than 30 % of the mean.

8.1.6 For the test portions obtained in 7.1.2 to 7.1.3, repeat steps 8.1.2 through 8.1.5 with calibrator solution C_1 (4.3.1), each in triplicate. For the test portion obtained in 7.1.4, repeat steps 8.1.2 through 8.1.5 with calibrator solution C_2 (4.3.2), each in triplicate. Calculate the mean calibrator C_1 or C_2 value (C_x). Repeat this determination if any one value varies by more than 30 % of the mean.

8.1.7 Calculate the correction value, C_r , using the mean calibration value, C_x (see 8.1.6), and the mean negative value, N (see 8.1.5) in Equation (1). Enter C_r in the luminometer. Consult the luminometer manual for instructions on how to change the luminometer correction to the new correction value number C_r :

$$C_r = (C_x - N) \times 0,286 \quad (1)$$

NOTE Automated luminometers will print N and C_x and automatically calculate and adjust the values of C_r and B_g .

8.1.8 Calculate the background value using C_r and the mean negative value N in Equation (2), and enter the value B_g in the luminometer. Consult the luminometer manual for instructions on how to change the luminometer background to the new background value number B_g :

$$B_g = [N/(C_r/100)] + 100 \quad (2)$$

NOTE Automated luminometers will print N and C_x and automatically calculate, adjust and print C_r and B_g .

8.1.9 Repeat steps 8.1.2 to 8.1.5 with the negative test sample (7.2), in triplicate. Verify whether the mean value with milk (in mU/l) is less than 5 mU/l, or in the case of flavoured-milk products and creams, less than 15 mU/l. If the mean value is out of range, go to step 8.1.1.

8.1.10 Repeat step 8.1.6 with calibrator C_1 or C_2 . The mean reading target range is between 320 mU/l and 400 mU/l for calibrator C (either C_1 or C_2). Repeat this determination if any one value varies by more than 30 % of the mean. If that value is out of range, go to step 8.1.1.

8.1.11 Perform the calibration assay (8.1.2 to 8.1.5) substituting in step 8.1.3 working calibrator A (A_1 or A_2 depending the test portion mentioned in 4.3) in place of the negative sample. Determine the mean value for calibrator A_1 or A_2 . Repeat this determination if any one value varies by more than 40 % of the mean value.

8.1.12 Perform the calibration assay (8.1.6) substituting working calibrator B (B_1 or B_2 depending the test portion mentioned in 4.3) in place of calibrator C in step 8.1.6. Determine the mean value for calibrator B. Repeat this determination if any one value varies by more than 30 % of the mean value.

8.1.13 The mean values for working calibrator A_1 shall be between 32 mU/l and 55 mU/l, and for working calibrator A_2 between 45 mU/l and 110 mU/l. The mean value for working calibrator B (either B_1 or B_2) shall be between 145 mU/l and 205 mU/l.

If both calibrators are in between the specified range, proceed to step 8.3. If either of the calibrators is out of the specified range, proceed to 8.2.1 and 8.2.2.

8.2 Calibration adjustment

8.2.1 Calibration adjustment for calibrators A and B

NOTE Automated calibration will make these calculations and adjustments and will prompt retest (8.2.2).

8.2.1.1 In cases where the mean value or mean values of calibrator A_1 , A_2 or B are slightly greater than or less (within 10 mU/l) than specified ranges, there may be an increase or decrease in background that will bring the mean value into range without causing the mean value of other calibrators to fall out of range (e.g. an increase in background will cause that amount to be subtracted from the mean value of each calibrator; a decrease in background will cause a corresponding increase to the mean value of each calibrator). In this case, change the luminometer background to this new background setting according to the following equations.

a) Use Equation (3) when calibrator A_1 is out of range:

$$32 \leq (A_{1m} - N_{A1}) \leq 55 \quad (3)$$

where

N_{A1} is the numerical value of the adjustment to bring the mean value of calibrator A_1 into range;

A_{1m} is the mean value of calibrator A_1 (calculated in 8.1.11);

32 is the lower limit value for calibrator A_1 ;

55 is the upper limit value for calibrator A_1 .

b) Use Equation (4) when calibrator A_2 is out of range:

$$45 \leq (A_{2m} - N_{A2}) \leq 110 \quad (4)$$

where

N_{A2} is the numerical value of the adjustment to bring the calibrator A_2 mean value into range;

A_{2m} is the mean value of calibrator A_2 ;

45 is the lower limit value for calibrator A_2 ;

110 is the upper limit value for calibrator A_2 .

c) Use Equation (5) when either calibrator B_1 or B_2 is out of range:

$$145 \leq (B_m - N_B) \leq 205 \quad (5)$$

where

N_B is the numerical value of the adjustment to bring the mean value of calibrator B_1 or B_2 into range;

B_m is the mean value of calibrator B_1 or B_2 (calculated in 8.1.12);

145 is the lower limit value for calibrator B_1 or B_2 ;

205 is the upper limit value for calibrator B_1 or B_2 .

After determining N_{AB} from Equation (3), (4) or (5), verify whether the other calibrator, X [calibrator B_1 or B_2 if Equation (3) or (4) was used, or calibrator A_1 or A_2 if Equation (5) was used], will stay in range, using Equations (6) and (7):

$$R_{x1} \leq X_{pro} \leq R_{x2} \quad \text{and} \quad (6)$$

$$X_{pro} = X_m - N_{AB} \quad (7)$$

where

R_{x1} is the lower limit of the mean of other calibrator X;

R_{x2} is the upper limit of the mean other calibrator X;

X_{pro} is the projected mean of calibrator X after N_{AB} adjustment;

X_m is the mean value of other calibrator X (calculated in 8.1.11 or 8.1.12);

N_{AB} is the numerical value of the adjustment for calibrator A_1 , A_2 , B_1 or B_2 calculated from Equation (3), (4) or (5).

If the calibrator, X, is in the specified range, then adjust the luminometer background value with the N_{AB} value to bring the calibrators into range according to Equation (8):

$$B_{g1} = B_g + N_{AB} \quad (8)$$

where

B_{g1} is the new luminometer background value;

B_g is the current luminometer background value.

8.2.1.2 In cases where the mean values of one calibrator are significantly outside the specified ranges (greater than 10), divide the obtained mean value of the calibrator concerned by its target values ($A_1 = 44$ mU/l or $A_2 = 88$ mU/l, and B_1 and $B_2 = 175$ mU/l).

Average the obtained two ratios for calibrators A and B to determine a mean correction ratio as show in Table 1.

Table 1 — Calibrator ratio

Calibrator	Target value	Mean value	Ratio (mean/target)
A ₁	44	A _{1m}	R _{A1} = A _{1m} / 44
A ₂	88	A _{2m}	R _{A2} = A _{2m} / 88
B ₁ or B ₂	175	B _{1m} or B _{2m}	R _B = B _{1m} or B _{2m} / 175
Mean ratio (R _m)			R _m = [(R _{A1} or R _{A2}) + R _B] / 2

Multiply the obtained mean ratio for calibrators A and B by the luminometer correction using Equation (9):

$$C_{r1} = C_r \times R_m \quad (9)$$

where

C_{r1} is the new luminometer correction;

C_r is the calculated luminometer correction (see 8.1.7);

R_m is the mean ratio of calibrators A and B (see Table 1);

Change the thus-obtained new luminometer correction to its new value, C_{r1}.

8.2.1.3 In cases where the mean value is significantly less (less than 10 units) than the target value and the other calibrator mean value is significantly greater (more than 10 units) than the target value, then determine the difference between the mean value of calibrator A (A₁ = 44 mU/l or A₂ = 88 mU/l).

Add this value to the background value of the luminometer; if the value is less than the target value, the difference is a negative number and the calculation should result in a decrease in background.

a) Calculate the new background value B_{g2} for calibrator A₁, by using Equation (10):

$$B_{g2} = B_g + (A_{1m} - 44) \quad (10)$$

where

B_g is the calculated luminometer background value (see 8.1.8)

B_{g2} is the new luminometer background value;

A_{1m} is the mean value of calibrator A₁ (see 8.1.11);

44 is the target value of calibrator A₁.

b) Calculate the new background value B_{g3} for calibrator A₂, by using Equation (11):

$$B_{g3} = B_g + (A_{2m} - 88) \quad (11)$$

where

B_g is the calculated luminometer background value (see 8.1.8)

B_{g3} is the new luminometer background value;

A_{2m} is the mean value of calibrator A₂ (see 8.1.11);

88 is the target value of calibrator A₂.

- c) Adjust the luminometer background setting as calculated. Reassay calibrator B and determine the mean value [$B_{m(1 \text{ or } 2)}$]. Divide the obtained mean by the target value (B_1 and $B_2 = 175$ mU/l). Multiply this ratio by the luminometer correction according to Equation (12):

$$C_{r2} = C_r \times \frac{B_{m(1 \text{ or } 2)}}{175} \quad (12)$$

where

- C_r is the calculated luminometer correction (see 8.1.7)
 C_{r2} is the new luminometer correction;
 $B_{m(1 \text{ or } 2)}$ is the mean value of calibrator B_1 or B_2 measured in the reassay;
 175 is the target value of calibrator B_1 or B_2 .

Adjust the luminometer correction to the new value.

8.2.2 Repeat steps 8.1.11 to 8.1.13. The mean readings of the luminometer shall be between 32 mU/l and 55 mU/l for working calibrator A_1 , and between 45 mU/l and 110 mU/l for working calibrator A_2 . Repeat the determination if any one value is greater or less than 40 % of the mean value.

The mean value for working calibrator B (either B_1 or B_2) shall be between 145 mU/l and 205 mU/l. Repeat the determination if any one value varies by more than 30 % of the mean value.

If both calibrator mean values are in range, follow step 8.3.3. If either calibrator mean value is still out of range, follow step 8.1.1.

8.3 Control tests and calibration check

8.3.1 Negative control test

Perform a negative control test according to the determination procedure (8.4) without adding the test portion (8.4.2). Perform a negative control test daily to verify the luminometer calibration and reagent performance. The system is verified (negative result) when the obtained alkaline phosphatase activity levels are less than 5 mU/l in the appropriately calibrated luminometer channel.

8.3.2 Positive control test

Rehydrate the positive control (4.4) with previously tested milk (dairy drink) showing a mean alkaline phosphatase activity of less than 5 mU/l (less than 15 mU/l with flavoured-milk products or cream products) or the prepared negative sample (7.2).

Perform a positive control test daily to verify the luminometer calibration and reagent performance. If the value determined exceeds 585 mU/l or is less than 300 mU/l, repeat with another rehydrated positive control. If the thus-obtained value is still out of range, recalibrate the luminometer (see 8.1).

8.3.3 Calibration check

8.3.3.1 After performing the calibration steps in 8.1 and 8.2, proceed with the negative control test (8.3.1) and triplicate determination of the positive control (8.3.2) to verify the calibration result.

8.3.3.2 The negative control shall give a value of less than 5 mU/l and the positive control ($N = 3$) mean value test shall be in the range 380 mU/l to 510 mU/l. If the negative control value is out of range, use a new substrate (4.1) and repeat the test. If the positive control value is out of range, or any individual value exceeds 585 mU/l or is less than 300 mU/l, check the temperature of the stopping solution (4.2) and repeat the test with another rehydrated positive control. If the thus-obtained value is still out of range on retests, recalibrate (see 8.1.1).

NOTE Triplicate determination of the positive control is used for verification of calibration. After calibration verification, only a single determination in the specified range is performed daily (see 8.3).

8.3.3.3 After calibrators and controls are in range, proceed with the determination (8.4). Up to 10 separate calibration channels can be set for different milk products in the luminometer (5.1). Test portions (7.1.2 and 7.1.4) should be read in the appropriate channel calibrated to that test portion. Raw milk (7.1.3) should be read in the channel calibrated for the test portion being used as diluent (7.2).

8.4 Determination

8.4.1 Limit the number to four vials per assay. Add, with the fixed volume pipette (5.3), 100 µl of the dioxetane ester substrate (4.1) to the test mini-vials (5.2), which are labelled on the upper half of the vial.

8.4.2 In the case of all test portions (7.1.1 to 7.1.4), add, with the fixed volume pipette (5.3) provided with a new tip, 100 µl of the prepared test portion to the contents of the mini-vials (8.4.1).

8.4.3 Mix the contents of vials in the vial rack with back and forth motion for about 10 times in 5 s. Place the mini-vials and contents (8.4.2) in the incubator block (5.7) set at 35 °C.

8.4.4 Select the appropriate luminometer channel calibrated for the test portion. After 3 min, add, with the fixed-volume dispenser (5.4), 1,0 ml of stopping solution (4.2) within 15 s.

8.4.5 Remove the mini-vials from the incubator. Close each vial with a cap. Shake each vial vigorously for 5 s. Uncap and attach the luminometer adapter, if necessary. Place each vial in the luminometer (5.1). Start the luminometer analysis and record readings after the luminometer beeps. Repeat the counting with each vial. Dilute any test portion resulting in a value higher than 7 000 mU/l and test it again.

NOTE When the enzyme reaction has stopped, the determination light output is stable at between 18 °C and 24 °C for 3 min. Luminometer readings are in mU/l enzyme units.

8.5 Heat-resistant microbial alkaline phosphatase control

If the determination (8.4) produces a positive result (≥ 350 mU/l is commonly used to identify incorrectly pasteurized samples), then proceed as follows.

Add 1 ml of another test portion (7.1.1 to 7.1.4) to a mini-vial and heat it in the incubator (5.7) or water bath (5.8) set at 63 °C. Keep it at this temperature for 30 min, and then cool it rapidly. Determine any residual phosphatase activity as specified in 8.4.

Any residual activity is due to the presence of heat-resistant microbial alkaline phosphatase. If the determination of the heated test sample is within 30 % of its original determination, then the entire phosphatase activity is microbial.

9 Calculation and expression of results

9.1 General

Results are calculated using software built into the luminometer (5.1). Calculations using readings from other luminometers may be performed manually using a hand-held calculator. If the results are to be calculated manually, proceed as given in 9.2.

9.2 Manual calculation

Record the RLU value (the relative light units) of the working calibrators A, B and C (4.3) and negative sample (7.2) and average the obtained results (8.1.1 to 8.1.13).

Calculate the alkaline phosphatase activity by using linear regression of the relative light units of the negative control and of calibrators A, B and C, and the associated target alkaline phosphatase activity (in mU/l) (e.g. negative control = 5 mU/l, $A_1 = 44$ mU/l or $A_2 = 88$ mU/l, B_1 and $B_2 = 175$ mU/l and C_1 and $C_2 = 350$ mU/l).

Use the average RLU value as the y co-ordinate and use the target mU/l value as the x co-ordinate. Calculate the slope m and the y -intercept b from the best linear fit according to Equation (13):

$$y = mx + b \quad (13)$$

where

x is the alkaline phosphatase activity of the sample, expressed in mU/l;

m is the numerical value of the slope of the regression line;

y is the numerical value of the RLU of the luminometer;

b is the numerical value of the intercept to obtain value of x .

Using the calculated m and b values, the enzyme unit determination of the test portion can be determined by using the measured RLU values as the y value and solving for x (mU/l) in Equation (14) as follows:

$$x = \frac{(y - b)}{m} \quad (14)$$

9.3 Expression of results

Express the test result to whole number of milliunits per litre (mU/l).

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are given in Annex B. The values for repeatability and reproducibility limit are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

The repeatability and reproducibility limits quoted in 10.2 and 10.3 apply at (average) enzyme levels of phosphatase activity of approx. 50 mU/l and 100 mU/l and ranging from 350 mU/l to 2 500 mU/l.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than:

- for enzyme levels of between 350 mU/l to 2 500 mU/l 21 % of the arithmetic mean;
- for enzyme levels approx. 100 mU/l 30 mU/l;
- for enzyme levels approx 50 mU/l 18 mU/l;
- for enzyme levels from negative samples 6 mU/l.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than:

- for enzyme levels of between 350 mU/l to 2 500 mU/l 41 % of the arithmetic mean;
- for enzyme levels approx. 100 mU/l 50 mU/l;
- for enzyme levels approx 50 mU/l 34 mU/l;
- for enzyme levels from negative samples 14 mU/l.

11 Test report

The test report shall specify:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the method used; with reference to this International Standard;
- d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents that may have influenced the test result(s);
- e) the test result(s) obtained or, if the repeatability has been checked, the final quoted results obtained.

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Annex A (informative)

Procedure flowchart

