



International Standard

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IDF 155-1

Milk and milk products — Determination of alkaline phosphatase activity —

Part 1: Fluorimetric method for milk and milk-based drinks

*Lait et produits laitiers — Détermination de l'activité de la
phosphatase alcaline —*

*Partie 1: Méthode fluorimétrique pour le lait et les boissons à
base de lait*

**Fourth edition
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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11

Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

International Dairy Federation
Silver Building • Bd Auguste Reyers 70/B
B-1030 Brussels
Phone: +32 2 325 67 40
Fax: +32 2 325 67 41
Email: info@fil-idf.org
Website: www.fil-idf.org

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Forewords

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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 302, *Milk and milk products — Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). It is being published jointly by ISO and IDF.

This fourth edition cancels and replaces the third edition (ISO 11816-1 | IDF 155-1:2013), which has been technically revised.

The main changes are as follows:

- the FLM200 (which has been discontinued) has been replaced by the FLM300 version;
- the instructions for use of the instrument and the flow of those instructions have been revised in accordance with FLM300, which has an upgraded user interface and electronics (there has been no change to the assay or the test procedure with the changes to the interface and software);
- the instrument now includes the heater block which was a separate item previously.

A list of all parts in the ISO 11816 | IDF 155 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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Milk and milk products — Determination of alkaline phosphatase activity —

Part 1: Fluorimetric method for milk and milk-based drinks

1 Scope

This document specifies a fluorimetric method for the determination of alkaline phosphatase (ALP) (EC 3.1.3.1) activity in raw and heat-treated whole milk, semi-skimmed milk, skimmed milk and flavoured milks.

This method is applicable to milk and milk-based drinks from cows, sheep and goats. It is also applicable to milk powder after reconstitution.

The instrument used for the determination of ALP can read activities up to 7 000 milliunits per litre (mU/l). If the activity is higher than 7 000 mU/l, it is diluted with ALP-free milk so as to obtain a measurement not higher than 7 000 mU/l.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1

alkaline phosphatase activity

ALP activity

activity of the enzyme present in the product, determined by the specified procedure

Note 1 to entry: The ALP activity is expressed as milliunits of enzyme activity per litre of sample (mU/l).

4 Principle

The ALP activity of the sample is measured by a continuous fluorimetric direct kinetic assay. A non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole phosphate, in the presence of any ALP derived from the sample, undergoes hydrolysis of its phosphate radical, producing a highly fluorescent product. Fluorimetric measurement of ALP activity is measured at

38 °C over a 3 min period when using a Fluorophos[®]¹⁾. This includes pre-incubation of substrate and sample, followed by multiple kinetic readings of the reaction rate.

NOTE Although this is a 3 min test, the first minute is an equilibration period to ensure that the sample is at 38 °C. Measurements of activity are actually made from the beginning of the second minute to the end of the third minute (i.e. over a 2 min period).

5 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

The reagents specified in 5.1 to 5.5 and the apparatus specified in 6.1 to 6.4 (except 6.3.3) comprise the Fluorophos[®] Test System²⁾. The manufacturer can change the packaging configurations supplied with Fluorophos[®] Test system. The user should refer to the manufacturer's instructions for preparing reagents if different from those specified herein.

5.1 Fluorophos[®] substrate, in bottles, each containing 144 mg of Fluorophos[®] substrate powder, molar mass of 580 g/mol.

This is a non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole phosphate (Fluorophos[®]).

The Fluorophos[®] substrate remains stable for two years from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C. Protect against light.

5.2 Substrate buffer solution, diethanolamine (DEA) buffer solution, $c(\text{DEA}) = 2,4 \text{ mol/l}$, with pH-value 10,0, in bottles of 240 ml each.

The substrate buffer solution remains stable for two years from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C. Protect against light.

5.3 Working substrate.

Allow the Fluorophos[®] substrate (5.1) and the substrate buffer solution (5.2) to come to room temperature. Add the content of one bottle of substrate buffer solution (240 ml) (5.2) to that of one bottle of Fluorophos[®] substrate (144 mg) (5.1) and mix well by inversion for 3 min to create an approximately 1,0 millimolar (pH-value 10) solution. Use amber glass to protect against light.

Allow the obtained solution to stand at room temperature for at least 30 min prior to use.

Use the analogue-to-digital (A/D) test given in 9.2 to test the suitability of the ready-to-use working substrate. Do not use the working substrate if a reading above 1 200 FLU (fluorescence units) is obtained.

The working substrate remains stable for 60 days when protected from light and stored at between 2 °C and 8 °C, or for 8 h when stored at 38 °C.

NOTE The volume of the working substrate (240 ml) obtained is sufficient for approximately 115 tests.

1) Fluorophos[®] is the registered trademark of a product supplied by Advanced Instruments, LLC. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

2) The Fluorophos[®] Test System is the trade name of a product supplied by Advanced Instruments, LLC. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.4 Calibrator solutions, Fluoroyellow^{®3)} (FY) [2'-(2-benzothiazolyl)-6'-hydroxybenzothiazole] in substrate buffer solution (5.2).

The calibrator solutions remain stable for 18 months from the date of manufacture, provided they are stored in unopened bottles at between 2 °C and 8 °C. Mix gently prior to use to ensure optimal results.

5.4.1 Calibrator solution A, containing 0 µmol/l of Fluoroyellow[®].

5.4.2 Calibrator solution B, containing $17,24 \times 10^{-3}$ µmol/l of Fluoroyellow[®].

5.4.3 Calibrator solution C, containing $34,48 \times 10^{-3}$ µmol/l of Fluoroyellow[®].

5.5 Daily instrument control solution, containing $34,48 \times 10^{-3}$ µmol/l of Fluoroyellow[®].

The daily instrument control solution remains stable for 18 months from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C. Mix gently prior to use to ensure optimal results.

6 Apparatus

Usual laboratory equipment and, in particular, the following shall be used.

6.1 Filter fluorimeter, with thermostatically controlled cuvette holder, capable of operating at $38\text{ °C} \pm 1\text{ °C}$ and right-angle optics, allowing excitation at a wavelength of 440 nm and emission between 520 nm and 560 nm [e.g. Fluorophos[®] instrument].

6.2 Cuvettes, disposable, non-fluorescent glass, of diameter 12 mm and of length 75 mm.

6.3 Pipettes.

6.3.1 Fixed-volume dispenser, capable of dispensing 2,0 ml.

6.3.2 Positive-displacement or air-displacement pipette, of capacity 0,075 ml.

Follow strict instructions for the pipetting technique as this is a critical step in generating accurate results. Ensure that the piston of the pipette bore is tightly secured prior to use.

6.3.3 Pipettes, of capacity 2 ml and 3 ml.

6.4 Incubator block, capable of maintaining a temperature of $38\text{ °C} \pm 1\text{ °C}$, suitable for holding cuvettes.

6.5 Plastic paraffin film, (e.g. Parafilm^{®4)}) or other suitable laboratory-grade film.

6.6 Vortex mixer.

6.7 Water bath, capable of maintaining a temperature of $63\text{ °C} \pm 1\text{ °C}$ and $95\text{ °C} \pm 1\text{ °C}$.

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

3) Fluoroyellow[®] is the registered trademark of a product supplied by Advanced Instruments, LLC. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the products named. Equivalent products may be used if they can be shown to lead to the same results.

4) Parafilm[®] is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this product.

7 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 document. A recommended sampling method is given in ISO 707 | IDF 50.

8 Preparations

8.1 Alkaline phosphatase-free milk

Prepare ALP-free milk of the type to be tested by carefully dispensing the desired portion of milk into a test tube or suitable container, ensuring that no milk touches the rim or sides of the container.

Place the tube or container with the milk portion in the water bath (6.7) set at 95 °C. Preheat the milk portion to 95 °C, before starting its 5 min heating period at that temperature. Check the temperature by using a thermometer or thermistor probe placed in the centre of the tube or container. When the milk portion reaches 95 °C, immediately start its 5 min heating period. Cool the whole portion rapidly after the heating period.

Test the thus-treated milk portion to ensure that its ALP activity is less than 10 mU/l.

8.2 Preparation of the test sample

8.2.1 General

Carefully mix all test samples prior to use.

NOTE It is usually not necessary to prewarm test samples.

8.2.2 Pasteurized test samples

Use pasteurized test samples as delivered, in amounts as required.

8.2.3 Dilution of test samples with high ALP values

Prepare dilutions of the test samples of milk using ALP-free milk (8.1) in order to bring their ALP levels within the analytical range of assay (< 7 000 mU/l). Mix the diluted solutions well.

9 Procedure

9.1 Verification of instrument performance

9.1.1 General

It is important to check instrument performance for drift, stray light and stability prior to analysing test samples. Follow good laboratory practice principles when operating the filter fluorimeter (6.1).

Quality control tests include the following:

- the daily A/D test, used to check the proper functioning of the equipment;
- the daily instrument control test, using the daily instrument control solution (5.5) to monitor any electronic or optical drift in the fluorimeter;
- the use of external positive, negative and normal controls, described in 9.1.3, which are recommended for monitoring daily instrument precision parameters.

9.1.2 Daily instrument tests

When using the Fluorophos® instrument, perform the A/D tests daily before testing commences.

9.1.3 Using FLM200

9.1.3.1 Access the A/D test according to the instrument's user guide through "SETUP" menu. Press "SETUP" key, then select menu items "A/D Test" by pressing < or > . With nothing in the cuvette holder, press "START". Allow the figures appearing on the display screen to stabilize. The display should read $302 \text{ FLU} \pm 4 \text{ FLU}$. If the reading is outside that range, clean the excitation and emission filters and repeat the A/D test.

9.1.3.2 Dispense 2,0 ml of daily instrument control solution (5.5) into a labelled cuvette. Place the cuvette in the incubator block (6.4) set at 38°C for 20 min. Insert the pre-warmed cuvette into the cuvette holder. Close the lid. When the display is stable, record the displayed value, which should be $602 \text{ FLU} \pm 12 \text{ FLU}$. If outside that range, use the small screwdriver supplied to slowly turn the potentiometer screw on the left-hand side of the instrument clockwise or anticlockwise, as necessary, until the display reads 602 FLU. Allow the numbers to equilibrate for 15 min.

9.1.4 Using FLM300

9.1.4.1 Access the A/D test according to the instrument's user guide through "SETUP" menu. With nothing in the cuvette holder, press "SETUP". Select "Diag.", by moving the [*] with the [< or >] keys and press "ENTER". A/D Test will be displayed. Press "START". Allow the figures appearing on the display screen to stabilize. The display should read 302 ± 4 . If the reading is outside that range, clean the excitation and emission filters and repeat the A/D test.

9.1.4.2 Dispense 2,0 ml of daily instrument control solution (5.5) into a labelled cuvette. Place the cuvette in the incubator block (6.4) set at 38°C for 20 min. Press "SETUP". Select "SETUP" by moving the [*] with the [< or >] keys and press "ENTER". "Daily Instrument Control Adj." will be scrolling across the screen. Press "START" to select it. Insert the cuvette labelled C1 into the instrument chamber, close the door, and press "ENTER". C1 shall read $602 \pm 12 \text{ FLU}$. If it does not meet this specification, move the [*] with the [< or >] keys to the right of "YES" to adjust it into the specification. If it does meet this specification, move the [*] with the [< or >] keys to the left of "NO" and press "ENTER".

9.1.5 Controls

Perform positive, negative and PhosphaCheck-N™ controls⁵⁾ using a powdered milk base with phosphatase and preservative.

The PhosphaCheck-N™ pasteurization controls remain stable for 18 months from the date of manufacture, provided they are stored in unopened and unreconstituted bottles at between 2°C and 8°C . Once reconstituted, the controls are stable for three days at between 2°C and 8°C . Do not freeze.

Allow the controls to come to room temperature. Reconstitute the PhosphaCheck® pasteurization controls before use. Remove the metal and rubber stopper. Add 3,0 ml of deionized water at room temperature.

Replace the stopper and mix gently by inversion for 1 min and then let stand for 15 min. Do not shake the controls or allow them to foam. Mix gently before each use to ensure optimal results.

After calibrating an unused channel with the negative control, analyse the three control solutions (i.e. positive, negative and PhosphaCheck-N™) by adding $75 \mu\text{l}$ of each control solution to 2 ml of pre-warmed substrate. Perform the ALP test.

5) The controls and instrument performance check instructions are products supplied by Advanced Instruments, LLC,. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the products named. Equivalent products may be used if they can be shown to lead to the same results.

The reading for the negative control shall be < 10 mU/l, the PhosphaCheck-N™ control shall be between 10 mU/l and 40 mU/l and the positive control shall be $500 \text{ mU/l} \pm 100 \text{ mU/l}$. These controls can be used daily to monitor the precision of the instrument.

9.2 Reagent controls to test the suitability of ready-to-use working substrate (5.3)

Dispense 2,0 ml of the working substrate (5.3) into a labelled cuvette. Place the cuvette in the heating block (6.4) set at 38 °C for 20 min. Insert the pre-warmed cuvette with the working substrate into the cuvette holder. Close the lid. When the display is stable, record the displayed value.

Freshly made substrate alone in the A/D mode usually gives a display reading of about 650 FLU which increases over time.

Do not use the working substrate when a display reading of above 1 200 FLU is obtained.

9.3 Calibration

9.3.1 General

Calibration curves are usually stable. However, recalibrate the instrument, which has already been calibrated, when the fluorimeter is initially installed, whenever servicing procedures (i.e. lamp or filter replacement) are likely to affect the stored calibration, or when assayed control values show unacceptable results.

If there are changes in the calibration curve, recalibrate the instrument using a new set of calibrator solutions A, B and C (5.4.1, 5.4.2 and 5.4.3). Establish a calibration curve for each type of product to be tested.

Mix calibrator solutions A, B and C by gentle inversion prior to use. Transfer, using the pipette (6.3.3), 2,0 ml of calibrator solution A, of calibrator solution B and of calibrator solution C (5.4.1, 5.4.2 and 5.4.3), respectively, each in duplicate, to six prelabelled cuvettes (6.2). Place the cuvettes in the incubator block (6.4) set at 38 °C and preheat for 20 min.

Add using the positive displacement or air-displacement pipette (6.3.2), 0,075 ml of ALP-free milk (see 8.1) to all six cuvettes. Cover the cuvettes with suitable laboratory-grade film (6.5). Mix their contents using the vortex mixer (6.6) for 5 s or by gently inverting the cuvettes. Return the cuvettes to the incubator block (6.4). Complete the calibration within 10 min after the addition of the test sample to the calibrator.

9.3.2 Using FLM200

9.3.2.1 Starting with calibrator solution A, perform the following calibration routine. Wipe the outside of each cuvette with soft tissue before placing the cuvette in the filter fluorometer (6.1). When using the FLM200 instrument, press "CALIB" and select the "ALP Dairy" menu. Scroll through the menu and press "ENTER" when the product to be calibrated is displayed. Beginning with calibrator solution A (5.4.1), insert this solution into the fluorimeter and press "START". When the measurement is finished, measure the second A calibrator solution.

9.3.2.2 Follow the same procedure for the B (5.4.2) and C (5.4.3) calibrator solutions until the procedure is completed. The Fluorophos® instrument automatically calculates the amount of fluorescence obtained with calibrator solution B and C against calibrator solution A to set the calibration ratio within the instrument.

Once calibration is completed, proceed to analyse the test samples.

9.3.3 Using FLM300

9.3.3.1 Starting with calibrator solution A, perform the following calibration routine. Wipe the outside of each cuvette with soft tissue before placing the cuvette in the filter fluorometer (6.1). When using the FLM300 instrument, press "CALIBRATE". Select "Milks or Other (mU/L)" with the [$<$ or $>$] keys and press "ENTER". Using the [$<$ or $>$] keys, select the channel to be calibrated and press "ENTER". Beginning with calibrator

solution A (5.4.1), insert this solution into the fluorimeter and press “START”. When the measurement is finished, measure the second A calibrator solution.

9.3.3.2 Follow the same procedure for the B (5.4.2) and C (5.4.3) calibrator solutions until the procedure is completed. The Fluorophos® instrument automatically calculates the amount of fluorescence obtained with calibrator solution B and C against calibrator solution A to set the calibration ratio within the instrument.

Once calibration is completed, proceed to analyse the test samples.

9.4 Determination

9.4.1 Dispense, using the fixed volume dispenser (6.3.1) or pipette, 2,0 ml of working substrate (5.3) into a labelled cuvette. Place the cuvette in the incubator block (6.4) set at 38 °C and heat for 20 min.

Add, using the pipette (6.3.2), 0,075 ml of the well-mixed test portion (see 8.2.2 or 8.2.3) to the substrate. Cover the cuvette with suitable laboratory-grade film (6.5). Immediately mix its contents using the vortex mixer (6.6) for 5 s or by gently inverting the cuvette. Wipe the outside of the cuvette with soft tissue and place it into the filter fluorimeter (6.1). The test shall be started within 20 s after the addition of the test portion to the substrate.

9.4.2 Using FLM200

When using the FLM200 instrument, press the “TEST” key. “ALP Dairy” appears, then press “ENTER”. Scroll through the menu and press “ENTER” when the product to be analysed is displayed. Then press the “START” key to begin the test and proceed to 9.4.4.

9.4.3 Using FLM300

When using the FLM300 instrument, press the “TEST” key. Select “Milks or Other (mU/L)” with the [< or >] keys and press “ENTER”. Using the [< or >] keys, select the product to be analysed and press “ENTER”. Enter an ID number and press “ENTER”. Press “START” to begin the test and proceed to 9.4.4.

9.4.4 Test for determination

The display will count down 60 s while the substrate and sample are being warmed to 38 °C. After 60 s, the fluorimeter starts measuring, displaying a fluorescence of the sample in FLU. The display starts at around 200 FLU and slowly increases over the next 2 min. At the end of the 3 min period, the Fluorophos® automatically performs the necessary calculations and displays the sample identification number, the ALP activity in milliunits per litre, and the average increase in fluorescence, if previously selected. This information will then be printed.

If results are to be calculated manually (see 10.2), divide the difference between the two fluorescence readings by the interval period (recorded in minutes) to obtain the average increase of fluorescence produced per minute (F/min). Use the F/min value to calculate the ALP activity of the test sample.

If the activity is higher than 7 000 mU/l, then dilute with the ALP-free milk (see 8.1) so as to obtain a measurement not higher than 7 000 mU/l.

It is possible that the Fluorophos® instrument displays and prints out the message “Error: Unstable Reading, Repeat Test”. For very low results (normally below 6 FLU/min), where the unstable readings are more common, leave the sample cuvette in the Fluorophos® chamber and perform another determination. A valid result is then usually obtained. If, however, an unstable reading error is obtained again, repeat the entire determination with a new test sample.

9.5 Test-sample-related controls

9.5.1 Recommended negative and positive control tests

9.5.1.1 Negative control test

Include a negative control test with each batch of test samples. Heat a test sample as described in [8.1](#). The instrument reading shall be less than 10 mU/l.

9.5.1.2 Positive control test

Include one or more positive controls with each batch of test samples. Prepare samples at or near decision levels using raw milk samples diluted with the ALP-free milk (see [8.1](#)).

9.5.2 Interfering substance test

Where higher than expected ALP values are obtained, add, using a pipette ([6.3.2](#)), 0,075 ml of test portion (see [8.2.2](#) or [8.2.3](#)) into a cuvette with 2,0 ml of calibrator solution A ([5.4.1](#)), which was previously pre-warmed in the incubator block ([6.4](#)) set at 38 °C for 20 min, and mix.

Place the cuvette containing this mixture in the instrument ([6.1](#)) and test as specified in [9.4](#). If the obtained value exceeds 20 mU/l, an interfering substance is shown to be present. In that case, repeat the test using a fresh sample.

9.5.3 Heat-stable microbial alkaline phosphatase control test

If the determination (see [9.4](#)) produces a higher result than the one expected, proceed as follows. Add another test portion (see [8.2.2](#) or [8.2.3](#)) into a tube. Place a thermometer or thermistor probe into the tube and put the whole portion in the water bath ([6.7](#)) set at 63 °C. When the test portion reaches 63 °C, keep it at that temperature for 30 min, then cool rapidly. Determine any residual phosphatase activity according to [9.4](#). Any residual activity is due to the presence of heat-stable microbial ALP.

10 Calculation and expression of results

10.1 Calibration ratio

Results are calculated automatically by the Fluorophos® instrument by means of the algorithm built into the filter fluorimeter ([6.1](#)). If the results are to be calculated manually, proceed as follows.

Record the fluorescence values of calibrator solution B ([5.4.2](#)) and calibrator solution C ([5.4.3](#)), read against calibrator solution A ([5.4.1](#)) set to zero fluorescence on the filter fluorimeter ([6.1](#)).

Calculate the calibration ratio, K , using [Formula \(1\)](#):

$$K = \frac{F_C + 2F_B}{4} \quad (1)$$

where

K is the numerical value of the calibration ratio of the established calibration curve;

F_C is the numerical value of the fluorescence obtained by measuring calibrator solution C ([5.4.3](#)) against calibrator solution A ([5.4.1](#)) set at zero fluorescence (see [9.3](#));

F_B is the numerical value of the fluorescence obtained by measuring calibrator solution B ([5.4.2](#)) against calibrator solution A ([5.4.1](#)) set at zero fluorescence (see [9.3](#)).

10.2 Calculation

Calculate the ALP activity, A_p , using [Formula \(2\)](#):

$$A_p = \frac{F_{av} \times c_B}{K \times V} \times f \quad (2)$$

where

- A_p is the numerical value of the ALP activity of the test sample (see [8.2.2](#) or [8.2.3](#)), in milliunits of enzyme activity per litre;
- F_{av} is the numerical value of the average amount of fluorescence produced per minute by the test portion (see [9.4](#)), measured against calibrator solution A (see [9.3](#)) currently from the beginning of the second minute to the end of the third minute;
- c_B is the concentration of the Fluoroyellow® in calibrator solution B ([5.4.2](#)), in micromoles per 2 ml of calibrator;
- f is the numerical value of the conversion factor from units per millilitre to milliunits per litre; $f = 1 \times 10^6$; in the case of samples diluted to obtain activities of not more than 7 000 mU/l, $f =$ (dilution factor of the test sample) $\times 10^6$;
- K is the numerical value of the calibration ratio of the established calibration curve;
- V is the numerical value of the volume of the test portion, in millilitres.

10.3 Expression of test results

Express the test results to the nearest whole unit of a milliunit. Results rounded to 1 mU/l are not required at all levels since once the result is above 200 mU/l the product has failed to pass the test and further accuracy is not required.

11 Precision

11.1 Interlaboratory study

The values for repeatability and reproducibility limits were derived from the results of collaborative trials carried out in accordance with ISO 5725-1 and ISO 5725-2. The values are expressed for the 95 % probability level and are not necessarily applicable to concentration ranges and matrices other than those given. Details of the interlaboratory study are summarized in [Annex A](#).

11.2 Repeatability

For values less than 125 mU/l, the absolute difference between two independent single test results, obtained with 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 14 mU/l.

For values 125 mU/l or higher and less than 620 mU/l, the absolute difference between two independent single test results, obtained with 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 12 % of the mean of the two determinations.

11.3 Reproducibility

For values less than 125 mU/l, the absolute difference between two single test results, obtained with 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 23 mU/l.

For values 125 mU/l or higher and less than 620 mU/l, the absolute difference between two single test results, obtained with 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 24 % of the mean of the two determinations.

12 Test report

The test report shall specify at least the following information:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, together with a reference to this document, i.e. ISO 11816-1 | IDF 155-1;
- d) all operating details not specified in this document, or regarded as optional, together with details of any incidents which can have influenced the test result(s);
- e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained;
- f) the date of the test.

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Annex A (informative)

Interlaboratory study

A collaborative trial, organized by QuadraChem Laboratories and Frank Harding, involving 13 laboratories from 7 countries (USA, UK, France, Norway, Italy, Netherlands and Switzerland) was carried out, in accordance with ISO 5725-1 and ISO 5725-2, on four types of cow's milk (whole, semi-skimmed, skimmed, flavoured) and on whole sheep's milk and whole goat's milk. The trial was completed in March 2004.

NOTE 1 Only flavoured milk, whole sheep's milk and whole goat's milk results are reported in this annex for the March 2004 trial. Results from cow's milk (whole, semi-skimmed and skimmed) are reported from subsequent trials (see below) completed in January 2008.

Another collaborative trial, organized by ANSES and Marina Nicolas, involving 19 laboratories from 18 countries (Northern Ireland, France, Austria, Switzerland, Hungary, Ireland, Norway, Germany, Finland, Belgium, Spain, Cyprus, Bulgaria, Portugal, Poland, Netherlands, Czech Republic and Greece) was carried out in accordance with ISO 5725-1 and ISO 5725-2 on cow's milk (whole, semi-skimmed and skimmed). The trial was completed in January 2008. Data from this trial replaces data from the 2004 trial.

Another collaborative trial, organized by ANSES and Marina Nicolas, was carried out in accordance with ISO 5725-1 and ISO 5725-2 on UHT semi-skimmed goat's milk. The trial was completed in December 2010. Data from this trial are reported below.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision determinations of [Clause 11](#). The actual average enzyme levels of the study sample are reported in [Table A.1](#). [Tables A.2](#) and [A.3](#) report repeatability and reproducibility limits. [Tables A.4](#) and [A.5](#) report the coefficients of variation of repeatability and reproducibility, respectively.

NOTE 2 Reference can be made to data from [Tables A.2](#), [A.3](#), [A.4](#) and [A.5](#) to monitor laboratory performance.

The overall report of the initial study was published in Reference [\[4\]](#) and the report of the additional trial was published by the European Union Reference Laboratory for Milk and Milk Products, ANSES (EX-AFSSA), French Food Safety Agency.

NOTE 3 There were insufficient data in some cases for cow's milk to calculate the $C_{V,r}$ (thus r) and $C_{V,R}$ (thus R) values at the 20 mU/l activity level. This was because the Fluorophos® instrument records a value of < 10 mU/l for very low ALP values and there is no approved statistical mechanism of dealing with such a result. This meant that all results correctly reported as < 10 mU/l had to be left out of statistical calculations.

Table A.1 — Enzyme mean values (mU/l) for each studied level in each matrix

Type of milk	Target enzyme level				
	20 (mU/l)	40 (mU/l)	100 (mU/l)	350 (mU/l)	500 (mU/l)
Whole cow's milk	24	40	120	350	488
Semi-skimmed cow's milk	27	40	124	345	479
Skimmed cow's milk	31	42	96	349	449
Flavoured cow's milk ^a	—	54	108	436	618
Whole sheep's milk	31	47	110	428	608
Whole goat's milk	22	47	125	407	570
Semi-skimmed goat's milk	29	57	110	317	474

^a The flavoured milk tested in this trial was strawberry.