
**Milk and milk products —
Determination of alkaline
phosphatase activity — Fluorimetric
microplate method**

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Forewords

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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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Milk and milk products — Determination of alkaline phosphatase activity — Fluorimetric microplate method

1 Scope

This document specifies a fluorimetric microplate method for the determination of alkaline phosphatase (ALP, EC 3.1.3.1)^[5] activity in raw and heat-treated whole milk, semi-skimmed milk, skimmed milk, cream, flavoured milks and cheeses.

This method is applicable to milk and milk-based drinks from cows, sheep and goats. Although the method was not tested in milk from other species, it can also be applicable to milk from other species with a similar composition to cow, sheep or goat milk, such as milk from buffalo and camelids. It is also applicable to milk powder after reconstitution and soft, semi-hard and hard cheeses provided that the mould is only on the surface of the cheese and not also in the inner part (e.g. blue veined cheeses). For large hard cheeses, specific conditions of sampling apply (see [Clause 7](#)).

NOTE This method was adapted from Reference [\[6\]](#).

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

amount of enzyme that is capable of catalysing the transformation of 1 μmol of substrate per minute under the conditions of the specified procedure

Note 1 to entry: The ALP activity in milk is expressed as milliunits of enzyme activity per litre of sample (mU/l) and as milliunits of enzyme activity per gram of sample (mU/g) in the case of cheese.

4 Principle

The ALP activity of the sample is measured by a continuous fluorimetric direct kinetic assay. A non-fluorescent aromatic monophosphoric substrate, 4-methylumbelliferone phosphate (4-MUP), in the presence of any ALP derived from the sample, undergoes hydrolysis of its phosphate, producing the highly fluorescent product 4-methylumbelliferone (4-MU). Fluorimetric measurement of ALP activity is measured at 37 °C, over a 15 min period.

The measured fluorescence is proportional to the concentration of the emitted fluorescent product and is used to calculate the enzyme activity.

5 Reagents

Use only reagents of recognized analytical grade.

5.1 Magnesium chloride solution, substance concentration, $c(\text{MgCl}_2) = 1 \text{ mol/l}$.

Weigh 50,8 g of magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, molecular mass is 203,3 g/mol) in a 50 ml glass beaker (6.17), dissolve with distilled water and transfer in a volumetric flask of 250 ml (6.18). Adjust the volume with water up to the mark.

This solution can be stored in aliquots (e.g. 10 ml) at below -20°C for one year.

5.2 Tergitol™ 15-S-9 (CAS Registry Number^{®1} 84133-50-6)²⁾ solution, $c = 100 \text{ g/l}$.

Weigh 25,0 g of Tergitol™ 15-S-9 in a 150 ml glass beaker (6.17), dissolve by stirring gently with distilled water at $25^\circ\text{C} \pm 2^\circ\text{C}$ and transfer in a volumetric flask of 250 ml (6.18). Adjust the volume with water up to the mark.

This solution can be stored in aliquots (e.g. 15 ml) at a temperature between 2°C and 8°C for one year.

5.3 2-amino-2-methyl-1-propanol (AMP) buffer solution, $c = 0,11 \text{ mol/l}$, $\text{pH} = 10,1$.

Weigh 9,8 g of AMP, purity $> 95\%$, molecular mass is 89,14 g/mol, in a 600 ml glass beaker (6.17), add 500 ml of distilled water on a stirrer and adjust pH to 10,1 with a hydrochloric acid solution $c(\text{HCl}) = 5 \text{ mol/l}$. Transfer in a 1 000 ml volumetric flask (6.18) and adjust with water up to the mark.

This solution can be stored in aliquots (e.g. 30 ml) at below -20°C for one year.

5.4 Diethanolamine (DEA) buffer solution, $c = 2 \text{ mol/l}$, $\text{pH} = 9,8$, $c(\text{Mg}^{2+}) = 0,5 \text{ mmol/l}$.

Weigh 210,3 g of DEA, molecular mass is 105,14 g/mol, in a 1 000 ml glass beaker (6.17), add 700 ml of distilled water while stirring and adjust pH to 9,8 with a hydrochloric acid solution $c(\text{HCl}) = 5 \text{ mol/l}$. Add 0,5 ml of MgCl_2 solution (5.1). Transfer in a 1 000 ml volumetric flask (6.18) and adjust with water up to the mark.

This solution can be stored in aliquots (e.g. 50 ml) at below -20°C for one year.

5.5 DEA extraction buffer, $c = 1,5 \text{ mol/l}$, $\text{pH} = 9,8$, $c(\text{Mg}^{2+}) = 1,5 \text{ mmol/l}$, $c(\text{Tergitol}^\text{TM}) = 0,1\%$.

Weigh 157,7 g of DEA in a 600 ml glass beaker (6.17), add 500 ml of distilled water on a stirrer and adjust pH to 9,8 with a hydrochloric acid solution $c(\text{HCl}) = 5 \text{ mol/l}$. Add 1,5 ml of MgCl_2 solution (5.1) and 10 ml of Tergitol™ 15-S-9 solution (5.2). Transfer in a 1 000 ml volumetric flask (6.18) and adjust with water up to the mark.

This solution can be stored in aliquots (e.g. 200 ml) at below -20°C for one year.

5.6 4-methylumbelliferone sodium salt (4-MU) standard stock solution, $c = 2,5 \text{ mmol/l}$.

Weigh 9,9 mg of 4-MU, purity $> 98\%$, molecular mass is 198,16 g/mol, in a 25 ml glass beaker (6.17), solubilize and rinse well with the AMP buffer solution (5.3), transfer to a volumetric flask of 20 ml (6.18) and fill up to the mark with the same buffer. Keep solution in the dark.

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This solution can be stored in aliquots (e.g. 200 µl) at below –20 °C for one year.

5.7 4-methylumbelliferone phosphate (4-MUP) substrate solution, $c = 2,5$ mmol/l.

Weigh 12,8 mg of 4-MUP, molecular mass is 256,15 g/mol, in a 25 ml glass beaker (6.17), solubilize and rinse well with the DEA buffer solution (5.4), transfer in a volumetric flask of 20 ml (6.18) and fill up to the mark with the same buffer.

Prepare the solution freshly and keep in the dark.

6 Apparatus and materials

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

6.1 Fluorescence microplate reader, capable of reading 96-well microplates at an excitation wavelength of 365 nm and emission wavelength of 450 nm. Capable of temperature control at $37\text{ °C} \pm 1\text{ °C}$ and allowing kinetic measurements (e.g. one reading per minute for 15 min).

6.2 Microplates, black, flat bottom, 96 well.

6.3 Pipette, of capacity 20 µl to 100 µl.

6.4 Pipette, of capacity 100 µl to 1 000 µl.

6.5 Multichannel pipettor, capable of dispensing 20 µl to 100 µl.

6.6 Air displacement pipette, of capacity 5 ml.

A glass pipette can also be used.

6.7 Microtubes, of capacity 2 ml.

6.8 Tubes, of capacity 15 ml and 50 ml.

6.9 Glass test tube, of approximately diameter 12 mm and length 10 cm.

6.10 Water bath, heating block or incubator suitable of maintaining a temperature of $37\text{ °C} \pm 1\text{ °C}$, $63\text{ °C} \pm 1\text{ °C}$ and $95\text{ °C} \pm 1\text{ °C}$.

6.11 Vortex mixer.

6.12 Parafilm^{®3)}, or other suitable laboratory-grade film.

6.13 Aluminium foil.

6.14 Grinding device.

6.15 ULTRA-TURRAX^{®4)}, or other homogenizer provided with a stem of diameter of approximately 6 mm to 8 mm.

3) 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.

4) ULTRA-TURRAX[®] 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.

6.16 Centrifuge, capable of centrifuging at 1 000*g* at 4 °C for 10 ml or 15 ml, and 50 ml tubes.

6.17 Glass beaker, of capacity 5 ml (approximately diameter 20 mm and length 30 mm), 10 ml (of approximately diameter 25 mm and length 30 mm), 25 ml, 50 ml, 150 ml, 600 ml and 1 000 ml.

6.18 One-mark volumetric flasks, of capacity 20 ml, 25 ml, 250 ml and 1 000 ml.

6.19 Analytical balance.

6.20 pH meter.

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^[1].

However, ISO 707 | IDF 50^[1] is not suitable for large hard cheeses where the whey curd mixture has been scalded at temperatures above 50 °C. If the cheese is made from raw milk, the ALP activity is not homogeneously distributed within these cheeses. The activity is high in the outer layer of the cheese wheel, between 0 cm to 4 cm below the rind of the round side, but very low or even undetectable in the core.

Samples of large hard cheeses, therefore, shall be sampled by taking a portion of 1 cm, taken at 0,5 cm below the rind of the round side (see [Figure C.1](#)).

In case of doubt regarding the type of cheese, between a hard and a semi-hard cheese, proceed to the sampling as described for large hard cheeses.

8 Preparation

8.1 Preparation of alkaline phosphatase-free sample

8.1.1 General

The ALP-free sample is used as sample blank, for calibration and for sample dilution if necessary.

8.1.2 Alkaline phosphatase-free milk

Prepare phosphatase-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. Cover the tube or container containing the milk portion and place it in the water bath or in the heating block ([6.10](#)) 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.

8.1.3 Alkaline phosphatase-free cheese

For each type of cheese to be tested, prepare a phosphatase-free cheese from the supernatant of the cheese (see [9.3.2.6](#)) by heating a portion of the supernatant as described in [8.1.2](#), replacing the milk by the supernatant.

8.2 Preparation of test sample

8.2.1 Milk samples

8.2.1.1 General

Carefully mix all test samples prior to use.

NOTE It is usually not necessary to prewarm test samples.

8.2.1.2 Pasteurized test samples

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

8.2.1.3 Dilution of test samples with high ALP values

Prepare dilutions of the test samples of milk using phosphatase-free milk (see [8.1.2](#)) in order to bring their ALP levels within the linearity range of the instrument (see [9.1.2](#)). Mix the diluted solutions well.

8.2.2 Cheese samples

8.2.2.1 General

Remove the rind or the surface from the test sample with a clean knife. Ensure that the test sample is not contaminated with surface microflora during its preparation. Especially for soft cheese with moulded surface, remove all the rind but in a layer as thin as possible, so as to avoid eliminating the fat layer under the mould surface (see [Annex C](#)). For large hard cheeses, proceed as described in [Clause 7](#). Grind the test sample by means of a grinding mill or other appropriate device ([6.14](#)) and mix thoroughly. Keep the prepared sample in an airtight container.

8.2.2.2 Dilution of test samples with high ALP values

Prepare dilutions of the supernatant of the cheese samples (see [9.3.2.6](#)) using phosphatase-free cheese (see [8.1.3](#)) in order to bring their ALP levels within the linearity range of the instrument (see [9.1.2](#)). Mix the diluted solutions well.

9 Procedure

9.1 Instrument

9.1.1 Instrument settings

Use equipment in accordance with the instructions provided by the manufacturer. The instrument ([6.1](#)) settings are adjusted according to [Table 1](#).

Set the photo multiplier tube (PMT) and optics according to the instrument manual (e.g. medium sensitivity, six readings per well).

To obtain the best fluorescence results with the instrument, the easiest way is to “scale to high well”. A good target for the high well is 80 % of the maximal signal before the detector saturates. After this adjustment, a blank reading should give no more than 10 % of the maximal signal.

Table 1 — Measurement settings

Measurement parameter	Instrument settings
Plate type	96 well, black, flat bottom
Operation mode	fluorescence type, kinetic 15 min, one reading per minute
Excitation wavelength	365 nm
Emission wavelength	450 nm
Temperature	37 °C
Reading action	shaking 5 s before the first read

9.1.2 Test the linearity range of the instrument

It is important to determine the linearity range of the instrument. This can be tested by ensuring that the measured relative fluorescent units (RFU) obtained with the standard curve follow a linear curve with a coefficient $R^2 \geq 0,99$.

9.1.3 Quality control

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) and refer to manufacturer's instructions.

9.2 Calibration

9.2.1 General

Establish a calibration curve for each type of product to be tested unless they have the same fat content. The concentrations given should be regarded as examples and should be adapted to the linearity range of the instrument (see 9.1.2).

9.2.2 Preparation of the working standard solutions

9.2.2.1 Prepare solutions freshly.

9.2.2.2 Dispense, using a pipette (6.4), 900 µl of AMP buffer solution (5.3) into a microtube (6.7). Add, using a pipette (6.3), 100 µl of 4-MU standard stock solution (5.6) so as to obtain a solution containing 250 µmol/l of 4-MU.

9.2.2.3 Introduce, using a pipette (6.4), 1 400 µl of the AMP buffer solution (5.3) in a microtube, labelled "A". Label six test microtubes (6.7) 1 to 6 and add, using pipette (6.4), 450 µl of the AMP buffer solution (5.3) in microtubes 2 to 6.

9.2.2.4 Add, using a pipette (6.3), 100 µl of the diluted standard stock solution (see 9.2.2.2) in microtube A. Mix thoroughly.

Perform serial dilutions similar to this example of solution (see 9.2.2.4) as followed and according to Table 2.

9.2.2.5 Draw, using a pipette (6.4), a first volume of 450 µl of the well mixed solution from microtube A in microtube 1 and a second volume of 450 µl of the well mixed solution from microtube A and add it to the 450 µl of AMP buffer solution (5.3) in microtube 2. Mix thoroughly. Repeat this process as necessary to achieve the desired concentration.

After mixing working standard solution number 6, remove 450 µl of the solution and discard before proceeding to 9.2.3.

Table 2 — Preparation of the 4-MU working standard solutions

Number of working standard solution	1	2	3	4	5	6
Concentration 4-MU working standard solutions, $\mu\text{mol/l}$	16,7	8,33	4,17	2,08	1,04	0,52
Volume AMP buffer solution (5.3), μl	/	450	450	450	450	450
Volume 4-MU/AMP mixed solutions, μl	450	450	450	450	450	450
Final volume in microtubes, μl	450	450	450	450	450	450



9.2.3 Preparation of the calibration curve

9.2.3.1 Add, using the pipette (6.3), 50 μl of phosphatase-free sample (see 8.1) to all six microtubes. Mix thoroughly.

9.2.3.2 Dispense, using the pipette (6.3), 100 μl of each standard solution, in triplicate, into the microplate (6.2). The final molarity per well is given in Table 3.

Keep standard samples in the dark until fluorescence measurement (see 9.3.3).

Table 3 — Final concentrations of 4-MU standard solutions

Number of standard solutions	1	2	3	4	5	6
Final concentration standard solutions, $\mu\text{mol/l}$	15,0	7,50	3,75	1,88	0,94	0,47
Volume 4-MU working standard solutions, μl	450	450	450	450	450	450
Volume ALP-free sample, μl	50	50	50	50	50	50
Transfer 100 μl of each standard solution in triplicate into the microplate						
Final molarity standard solutions, $\mu\text{mol/well}$	$1,5 \times 10^{-3}$	$7,5 \times 10^{-4}$	$3,75 \times 10^{-4}$	$1,88 \times 10^{-4}$	$9,38 \times 10^{-5}$	$4,69 \times 10^{-5}$

9.3 Determination

9.3.1 Milk samples

9.3.1.1 Dispense, using the pipette (6.4), 450 μl of AMP buffer solution (5.3) into a labelled microtube (6.7).

9.3.1.2 Add, using the pipette (6.4), 50 μl of the well-mixed test sample (see 8.2.1) to the buffer. Cover the microtube. Immediately mix its contents using the vortex mixer (6.11) for 5 s or by gently inverting the microtube.

9.3.1.3 Introduce, using the pipette (6.3), 100 μl of the preparation (see 9.3.1.2), in triplicate, into the microplate (6.2).

9.3.1.4 Perform the fluorescence measurement (see [9.3.3](#)).

9.3.2 Cheese samples

9.3.2.1 Weigh, to the nearest 1 mg, 0,3 g to 0,5 g of the prepared test sample (see [8.2.2.1](#)) into a 10 ml glass beaker ([6.17](#)) or into a glass test tube ([6.9](#)).

9.3.2.2 Add a first portion of 5 ml of DEA extraction buffer ([5.5](#)). Let the DEA extraction buffer be in contact with the cheese between approximately 5 min and 10 min for soft and semi-hard cheese and between 15 min and 20 min for hard cheese. Homogenize the buffer/cheese mixture using an ULTRA-TURRAX® or a homogenizer ([6.15](#)) until complete dissolution of the test sample (approximately 35 s for soft and semi-hard cheeses and 50 s for hard cheese). Transfer this first buffer/cheese mixture quantitatively into 25 ml one-mark volumetric flask ([6.18](#)).

9.3.2.3 Add a second portion of 5 ml of DEA extraction buffer ([5.5](#)) while rinsing the ULTRA-TURRAX®/homogenizer stem and mix approximately for 30 s. Transfer the buffer/cheese mixture quantitatively into the 25 ml one-mark volumetric flask.

9.3.2.4 Rinse the ULTRA-TURRAX®/homogenizer stem and the beaker with a new portion of 5 ml of DEA extraction buffer ([5.5](#)) and transfer to the 25 ml volumetric flask. Dilute to the 25 ml mark with DEA extraction buffer ([5.5](#)) and shake gently.

9.3.2.5 Introduce the final buffer/cheese mixture in a 50 ml test tube ([6.8](#)) for centrifuge. Centrifuge at 1 000g at 4 °C during 10 min to remove the fat. After centrifugation, three more or less distinct layers are obtained: a pellet, a supernatant and the surface fat.

9.3.2.6 Incline the test tube and introduce the pipette ([6.6](#)) into the supernatant. Carefully remove the supernatant as much as possible with the pipette and transfer it in a 50 ml glass beaker ([6.17](#)) or a tube ([6.8](#)). From this point on, measurements carried out (see [9.3.2.7](#) to [9.3.2.10](#) and [9.3.3](#)) are done in the same way as for the determination of ALP activity in milk samples (see [9.3.1](#)), replacing the milk sample by the supernatant (see [9.3.2.6](#)).

9.3.2.7 Dispense, using the pipette ([6.4](#)), 450 µl of AMP buffer solution ([5.3](#)) into a labelled microtube ([6.7](#)).

9.3.2.8 Add, using a pipette ([6.3](#)), 50 µl of the supernatant (see [9.3.2.6](#)) to the buffer. Cover the microtube. Immediately mix its contents using the vortex mixer ([6.11](#)) for 5 s or by gently inverting the microtube.

9.3.2.9 Introduce, using the pipette ([6.3](#)), 100 µl of the preparation (see [9.3.2.8](#)), in triplicate, into the microplate ([6.2](#)).

9.3.2.10 Perform the fluorescence measurement.

9.3.3 Plate layout and fluorescence measurement

9.3.3.1 An example of a microplate layout containing 21 samples is given in [Table 4](#).

Table 4 — Example of an assay layout for a 96-well microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Std 2	Std 2	Std 2	Std 3	Std 3	Std 3	Std 4	Std 4	Std 4
B	Std 5	Std 5	Std 5	Std 6	Std 6	Std 6	ALP FS	ALP FS	ALP FS	Pos	Pos	Pos
C	Neg	Neg	Neg	U1	U1	U1	U2	U2	U2	U3	U3	U3
D	U4	U4	U4	U5	U5	U5	U6	U6	U6	U7	U7	U7
E	U8	U8	U8	U9	U9	U9	U10	U10	U10	U11	U11	U11
F	U12	U12	U12	U13	U13	U13	U14	U14	U14	U15	U15	U15
G	U16	U16	U16	U17	U17	U17	U18	U18	U18	U19	U19	U19
H	U18	U18	U18	U19	U19	U19	U20	U20	U20	U21	U21	U21
Key Std standard ALP FS ALP-free sample Pos positive Neg negative U unknown sample												

9.3.3.2 Place the microplate in the filter fluorimeter ([6.1](#)), or in the incubator ([6.10](#)) set at 37 °C, for 5 min.

9.3.3.3 Dispense, using the multi-channel pipettor ([6.5](#)), 100 µl of 4-MUP substrate solution ([5.7](#)) to all standard, control and sample wells.

9.3.3.4 Transfer the uncovered microplate to the filter fluorimeter ([6.1](#)), previously set at 37 °C.

9.3.3.5 Measure initial fluorescence at the start of the reaction and subsequently every minute for 15 min. Start the test immediately after the addition of the substrate.

If the relative fluorescence at the end of the measurement is higher than the relative fluorescence of the highest standard (e.g. 25 µmol/l), the samples shall be diluted with the ALP free sample (see [8.1](#)) so as to obtain a measurement not higher than the highest standard.

9.4 Test sample-related controls

9.4.1 Recommended negative and positive controls

9.4.1.1 Negative control test

Include a negative control test with each batch of test samples.

For milk samples, heat a test sample as described in [8.1.2](#). The value of the ALP activity shall be less than 15 mU/l.

For cheese samples, include a cheese made from pasteurized milk as the negative control test. The value of the ALP activity shall be less than 15 mU/g.

RFU values of the negative control test can vary depending on the gain setting. However, these values shall remain constant during the measuring period.

9.4.1.2 Positive control test

Include one or more positive controls with each batch of test samples.

For milk samples, prepare samples at or near decision levels using raw milk samples diluted with the phosphatase-free milk (see [8.1.2](#)).

9.4.2 Interfering substance test

Where higher than expected ALP values are obtained, perform a sample measurement without addition of substrate by adding, using a pipette ([6.3](#)), 0,1 ml of test portion (see [9.3.1.3](#) or [9.3.2.9](#)) into a microplate ([6.2](#)) and incubate at 37 °C for 5 min. Subsequently, add 0,1 ml of DEA buffer solution ([5.4](#)), mix and start the analysis as described (see [9.3.3.4](#) and [9.3.3.5](#)).

If the obtained value exceeds 20 mU/l, an interfering substance is present. In that case, repeat the test using a fresh sample.

9.4.3 Heat-stable microbial alkaline phosphatase control test

The control test for microbial ALP is recommended, if the determination (see [9.3](#)) produces a higher result than the one expected.

However, the test is compulsory if in cheeses produced with pasteurized milk, the determination (see [9.3.2](#)) produces a positive result.

Introduce another test portion of the milk sample (see [8.2.1](#)) or the supernatant (see [9.3.2.6](#)) into a tube. Place a thermometer or thermistor probe into the tube and put the whole in the water bath ([6.10](#)) maintained 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 [9.3](#). Any residual activity is due to the presence of heat-stable microbial ALP.

10 Calculation and expression of results

10.1 Calibration curve

Determine the linearity of the calibration curve. Samples should be measured in the linear part of the curve. Samples with values above the value of the highest standard shall be diluted to achieve this. The linear part of the curve is dependent upon the apparatus used and its settings.

Check that ALP activity of the ALP free sample (see [8.1](#)) is less than 15 mU/l.

Using the six standard solutions (see [9.2.3](#)), prepare a calibration curve by plotting the 4-MU standards molarity ($\mu\text{mol/well}$) versus RFU average of the triplicates obtained for the zero time point.

Determine the equation of the line using least squares regression and the fluorescence slope of the calibration curve (Δ_{std}).

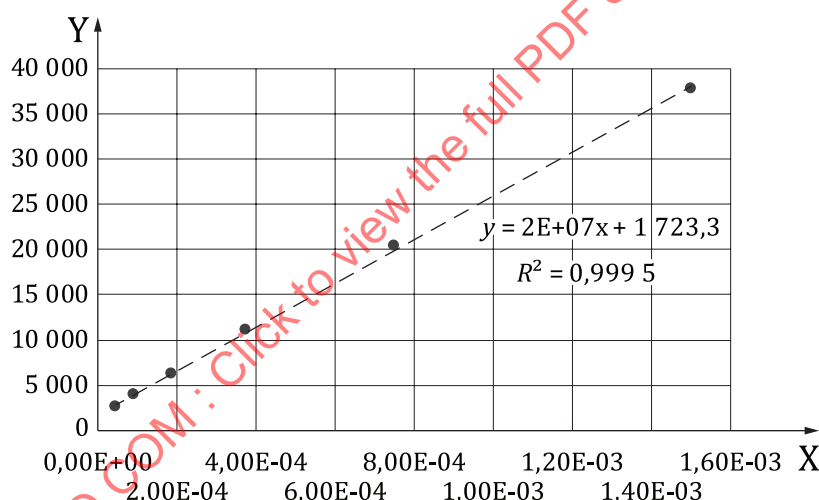
An example of a calibration curve is presented in [Table 5](#) and [Figure 1](#).

Table 5 — Example of a 4-MU standard curve calculation

4-MU standard solutions μmol/l	x-axis μmol/well	y-axis RFU average
15	$1,50 \times 10^{-3}$	37 695,0
7,5	$7,50 \times 10^{-4}$	10 319,7
3,75	$3,75 \times 10^{-4}$	11 064,7
1,875	$1,88 \times 10^{-4}$	6 143,0
0,9 375	$9,38 \times 10^{-5}$	3 912,3
0,46 875	$4,69 \times 10^{-5}$	2 571,6
	Slope	24 166 413

The left-hand column in [Table 5](#) gives the concentrations of the 4-MU standard solutions in μmol/l. The middle column gives the amount of substrate per well in μmol, which is represented on the x-axis of [Figure 1](#). The right-hand column gives an example of average RFU measured, which is represented on the y-axis of [Figure 1](#). [Table 5](#) gives the slope of the linear curve (lower right field).

[Figure 1](#) is an example graph obtained based on the standards and the results as described in [Table 5](#). The number of μmols of substrate are shown on the x-axis, the RFU are shown on the y-axis and the resulting linear equation with regression coefficient are indicated.



Key

X μmol/well
Y RFU

Figure 1 — Example graph of a 4-MU standard curve

10.2 Calculation of ALP activity of unknown samples

10.2.1 Milk samples

Calculate the average slope of the time-dependent fluorescence increase of the sample, Δ_S and the blank, Δ_B , using linear regression.

Calculate the ALP activity, A , using [Formula \(1\)](#):

$$A = \frac{\Delta_S - \Delta_B}{\Delta_{Std}} \times \frac{f_1}{V} \times f_2 \quad (1)$$

where

- A is the numerical value of the ALP activity of the test sample (see 8.2.1), in milliunits of enzyme activity per litre (mU/l);
- Δ_S is the fluorescence slope of the sample, in RFU/min;
- Δ_B is the fluorescence slope of the blank, in RFU/min;
- Δ_{Std} is the fluorescence slope of the calibration curve, in RFU/ μ mol;
- f_1 is the numerical value of the conversion factor from units per litre to milliunits per litre;
 $f_1 = 1\ 000$;
- V is the numerical value of the volume of the test portion, in litres, $(100 \times 10^{-6})/10$;
- f_2 is the numerical value of the dilution factor, if any, of the test sample to obtain an activity detection value not higher than the highest standard.

10.2.2 Cheese samples

10.2.2.1 Supernatant

Calculate the average slope of the time-dependent fluorescence increase of the sample, Δ_S and the blank, Δ_B , using linear regression.

Calculate the ALP activity of the supernatant, A_1 , (see 9.3.2.6) using Formula (2):

$$A_1 = \frac{\Delta_S - \Delta_B}{\Delta_{Std}} \times \frac{f_1}{V} \quad (2)$$

where

- A_1 is the numerical value of the ALP activity of the supernatant (see 9.3.2.6), in milliunits of enzyme activity per litre (mU/l);
- Δ_S is the fluorescence slope of the supernatant, in RFU/min;
- Δ_B is the fluorescence slope of the sample blank, in RFU/min;
- Δ_{Std} is the fluorescence slope of the calibration curve, in RFU/ μ mol;
- f_1 is the numerical value of the conversion factor from units per litre to milliunits per litre;
 $f_1 = 1\ 000$;
- V is the numerical value of the volume of the test portion, in litres, $(100 \times 10^{-6})/10$.

10.2.2.2 Cheese

Calculate the ALP activity of the test sample, A , using Formula (3):

$$A = \frac{A_1 \times 25 \times f_2}{1\ 000 \times m} \quad (3)$$

where

- A is the numerical value of the ALP activity of the test sample, in milliunits of enzyme activity per gram (mU/g);
- A_1 is the numerical value of the ALP activity of the supernatant (see 9.3.2.6), in milliunits of enzyme activity per litre (mU/l);
- f_2 is the numerical value of the dilution factor, if any, of the initial supernatant to obtain an activity detection value not higher than the highest standard;
- m is the mass, in grams, of the test portion in the 25 ml one-mark volumetric flask (6.18).

10.2.3 Calculation example

Table 6 gives an example of an ALP activity calculation. The RFU measured over 15 min are listed for an ALP free sample (see 9.4.1.1), positive control (see 9.4.1.2) and an unknown sample in triplicate. The slope for each sample is calculated using linear regression (see 10.2.2.1) and the ALP activity in mU/l is calculated according to Formulae (3), (4) and (5).

Figure 2 gives an example graph of ALP activity based on the values shown in Table 6.

Table 6 — Examples of the calculation of ALP activity in controls and unknown samples

Time min	ALP free sample	Positive control 1:100	Negative control 1:1	Unknown sample 1	Unknown sample 2	Unknown sample 3
0	1 491	2 892	1 932	3 644	2 080	1 747
1	1 507	5 056	1 939	6 136	2 861	2 002
2	1 499	7 278	1 945	8 651	3 674	2 228
3	1 508	9 339	1 947	11 091	4 393	2 415
4	1 511	11 512	1 950	13 574	5 195	2 632
5	1 513	13 614	1 951	16 076	5 960	2 848
6	1 509	15 741	1 982	18 614	6 765	3 077
7	1 491	17 651	1 984	20 867	7 468	3 258
8	1 505	19 768	1 980	23 281	8 264	3 475
9	1 515	21 705	1 992	25 447	8 986	3 681
10	1 522	23 790	1 985	27 905	9 775	3 908
11	1 490	25 826	1 986	30 283	10 472	4 100
12	1 503	27 670	1 989	32 491	11 239	4 294
13	1 517	29 693	2 016	34 871	12 004	4 515
14	1 515	31 740	2 028	37 262	12 821	4 711
15	1 493	33 443	2 031	39 419	13 487	4 922
Slope A_B, A_S	0,32	2 041	6,27	2 384	761	210
Slope (A_{Std})		24 166 413	24 166 413	24 166 413	24 166 413	24 166 413
Conversion factor, f_1		1	1	1	1	1
Dilution factor, f_2		100	1	50	1	1
Volume of the portion, V		0,000 01	0,000 01	0,000 01	0,000 01	0,000 01
Activity, mswU/l		844 497,7	25,9	493 301,8	3 147,9	867,3

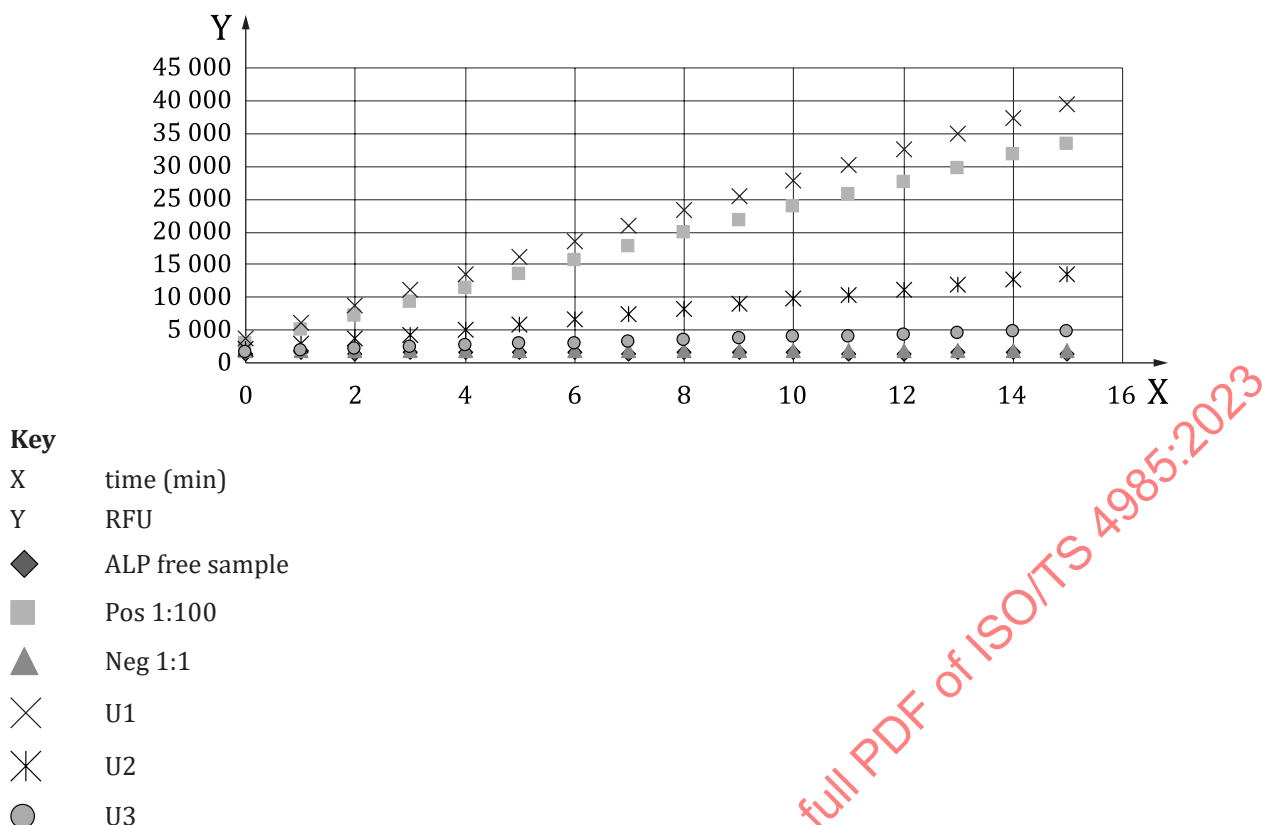


Figure 2 — Example graph of ALP activity of controls and unknown samples

10.3 Expression of results

Express the test results to the nearest whole unit of a milliunit. Results below the detection limit of 15 mU/l should be reported as < 15 mU/l.

11 Precision

11.1 Interlaboratory test

The values for repeatability and reproducibility limits were derived from the results of interlaboratory tests carried out in accordance with ISO 5725-1^[2] and ISO 5725-2^[3], see [Annex A](#). The values are expressed for the 95 % probability level and are not necessarily applicable to concentration ranges and matrices other than those given.

11.2 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 the cases be greater than 4,1 % of the mean of the two determinations.

11.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of the cases be greater than 12,8 % of the mean of the two determinations.

12 Test report

The test report shall specify 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 reference to this document, i.e. ISO 4985 | IDF 255:2023;
- 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)

Precision data

The data given in [Table A.1](#) were obtained in two interlaboratory studies organized by ANSES and conducted in accordance with the ISO 5725-1[2] and ISO 5725-2[3] for collaborative study procedures to validate characteristics of a method of analysis.

A first pilot trial, involving five laboratories from five countries (Belgium, France, Ireland, Netherlands and Switzerland) was carried out in April 2017 on milk and one cheese from cow milk.

A second collaborative trial, involving four laboratories from four countries (France, Ireland, Netherlands and Switzerland) was organized in September 2017 on cow's milk. The results of the interlaboratory trials were published in Reference [7]. A method comparison between the microplate procedure given in this document and the reference method (Fluorophos) is given in [Annex B](#).

Table A.1 — Statistical parameters interlaboratory study

Sample	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h
Date of interlaboratory test	April 2017	April 2017	April 2017	April 2017	April 2017	Sept 2017	Sept 2017	Sept 2017
Number of participating laboratories	5	5	5	5	5	4	4	4
Number of outliers	0	0	0	1	0	0	0	0
Number of accepted results	5	5	5	5	5	4	4	4
Mean value, $\bar{X}$, mU/g	< 15	425	5 526	112	121	< 15	115	361
Repeatability standard deviation s_r , mU/l; mU/g	—	7,4	180	4,1	2,6	—	3,4	10,4
Repeatability coefficient of variation, $C_{V,r}$, %	—	1,7	3,2	3,7	2,2	—	3,4	10,4
Repeatability limit r [$r = 2,8 \times s_r$], mU/l; mU/g	—	20,6	503,5	11,4	7,4	—	9,5	29,0
Repeatability relative, %	—	4,8	9,0	10,3	6,2	—	8,2	8,0
Reproducibility standard deviation s_R , mU/g	—	65,9	835,4	15,7	12,4	—	13,5	38,7
Reproducibility coefficient of variation, $C_{V,R}$, %	—	15,5	14,9	14,1	10,4	—	11,8	10,7
Reproducibility limit R [$R = 2,8 \times s_R$], mU/g	—	184,6	2 339	43,8	34,8	—	37,8	108,4
Reproducibility relative, %	—	43,4	41,7	39,5	29,2	—	32,9	30,1

^a Negative whole cow milk, ^b Whole cow milk target "350 mU/l" Fluorophos, ^c Raw cow milk, ^d Mixed cheese, ^e Cheese supernatant, ^f Negative whole cow milk, ^g Whole cow milk target "100 mU/l" Fluorophos, ^h Whole cow milk target "350 mU/l" Fluorophos