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EAS 160 (2000) (English): Milk and dried milk, butter milk and butter milk powder, whey, and whey powder—  
Determination of phosphatase activity



**ISO INSIDE**



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**EAS 160:2000**  
**ICS 67.100.10**

## **EAST AFRICAN STANDARD**

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**Milk and dried milk, butter milk and butter milk powder, whey and whey powder — Determination of phosphatase activity**

**EAST AFRICAN COMMUNITY**

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# EAS 160:2000

## Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in East Africa. It is envisaged that through harmonized standardization, trade barriers which are encountered when goods and services are exchanged within the Community will be removed.

In order to achieve this objective, the Partner States in the Community through their National Bureaux of Standards, have established an East African Standards Committee.

The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the private sectors and consumer organizations. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the procedures of the Community.

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

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East African Community

P O Box 1096

**Arusha**

Tanzania

Tel: 255 27 2504253/8

Fax: 255-27-2504481/2504255

E-Mail: [eac@eachq.org](mailto:eac@eachq.org)

Web: [www.each.org](http://www.each.org)

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TECHNICAL  
SPECIFICATION

ISO/TS  
6090

IDF/RM  
82

First edition  
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**Milk and dried milk, buttermilk and  
buttermilk powder, whey and whey  
powder — Detection of phosphatase  
activity**

*Lait et lait sec, babeurre et babeurre en poudre, lactosérum et  
lactosérum en poudre — Détermination de l'activité phosphatasique*



Reference numbers  
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IDF/RM 82:2004(E)

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Case postale 56 • CH-1211 Geneva 20  
Tel. + 41 22 749 01 11  
Fax + 41 22 749 09 47  
E-mail [copyright@iso.org](mailto:copyright@iso.org)  
Web [www.iso.org](http://www.iso.org)

International Dairy Federation  
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels  
Tel. + 32 2 733 98 88  
Fax + 32 2 733 04 13  
E-mail [info@fil-idf.org](mailto:info@fil-idf.org)  
Web [www.fil-idf.org](http://www.fil-idf.org)

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## 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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
- an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

ISO/TS 6090|IDF/RM 82 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 AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

## 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 and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

The main task of technical committees is to prepare International Standards. 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 IDF National Committees casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish an other type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

ISO/TS 6090|IDF/RM 82 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 AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Characterization of heat treatment*, of the Standing Committee, *Minor components and characterization of physical properties*, under the aegis of its project leader, Mr E. Wolters (DK).



## **Introduction**

This Technical Specification specifies a method which is not affected by the presence of phenol and is of particular use where difficulties due to environmental contamination by traces of phenol can give false results by the method as specified in ISO 3356. It is possible to detect a mass fraction of 0,5 % raw milk in pasteurized milk by procedure A, and a mass fraction of 0,2 % raw reconstituted product in pasteurized reconstituted product by procedure B.

The method is published as a Technical Specification rather than an International Standard since no interlaboratory trial of the method could be organized fulfilling the requirements of ISO 5725-1 and ISO 5725-2.



# Milk and dried milk, buttermilk and buttermilk powder, whey and whey powder — Detection of phosphatase activity

## 1 Scope

This Technical Specification specifies a screening method for the detection of the phosphatase activity in cow's milk and dried milk, buttermilk and buttermilk powder, and whey and whey powder.

Two alternative procedures (A and B) are given. Procedure A is the simpler as it does not include a clarification treatment and is especially suited for milk. Procedure B includes a clarification step which allows a more sensitive detection through which better quantitative results can be obtained, if desired.

If either method is used for checking the proper pasteurization of these products or their raw materials, then further tests are necessary to ensure that the phosphatase activity is not due to either heat-stable microbial phosphatase (see 9.3.1) or reactivated phosphatase (see 9.3.2).

NOTE A method using a patented proprietary reagent is described in Annex A.

## 2 Normative references

The following referenced document is indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3356, *Milk and dried milk, buttermilk and buttermilk powder, whey and whey powder — Determination of phosphatase activity (Reference method)*<sup>1)</sup>

## 3 Terms and definitions

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

### 3.1

#### **phosphatase activity**

activity of the (undenatured) alkaline phosphatase determined by the procedures described in this Technical Specification

NOTE The phosphatase activity is expressed as a negative or positive result, and/or “to be re-examined according to ISO 3356”.

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1) Equivalent to IDF 63.

## 4 Principle

The sample of the liquid product or that of the reconstituted liquid product is diluted with a buffer substrate containing disodium *p*-nitrophenyl phosphate at pH 10,6 and incubated at 37 °C for 2 h. The *p*-nitrophenol is liberated by hydrolysis under the influence of any active alkaline phosphatase present in the sample. Any liberated *p*-nitrophenol is detected directly (method A) or after clarification (method B) by its yellow colour, either visually or spectrometrically. If a positive result is obtained, the sample is tested for the presence of heat-stable microbial phosphatase via pasteurization, or for the presence of reactivated phosphatase via a dilution of the sample to which magnesium acetate solution is added to reactivate the phosphatase. Either method A or method B is used to detect their presence.

## 5 Reagents

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

**5.1 Sodium hydroxide solution**,  $c(\text{NaOH}) = 0,5 \text{ mol/l}$  or  $0,1 \text{ mol/l}$ .

**5.2 Diethanolamine hydrochloride buffer solution**, pH = 10,6.

Dissolve 15,8 g of diethanolamine hydrochloride  $[(\text{C}_2\text{H}_5\text{O})_2\text{NH}_2\text{Cl}]$  in water. Dilute to 1 000 ml with water.

**5.3 Buffer substrate solution**

Dissolve 1,5 g of disodium *p*-nitrophenol phosphate or 1,3 g of dihydrogen *p*-nitrophenol phosphate in diethanolamine hydrochloride buffer solution (5.2). Dilute to 1 000 ml with the buffer solution.

If stored in a refrigerator at 4 °C or lower, the buffer substrate solution is stable for up to four weeks. If 0,1 % chloroform ( $\text{CHCl}_3$ ) is added, the buffer substrate solution can be kept for three to four months at that temperature.

Any instability is denoted by the formation of a yellow colour. The test is always carried out with reference to a boiled product control containing the same quantities of buffer substrate solution. It is recommended, therefore, to prepare a new solution if the present gives a colour reading in excess of 10 µg when read in a 25 mm cell in the comparator (6.3) using distilled water in the other 25 mm cell.

**5.4 Precipitants**

**5.4.1 Zinc sulfate solution**

Dissolve 30 g of zinc sulfate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) in water. Dilute to 100 ml with water.

**5.4.2 Potassium hexacyanoferrate(II) solution**

Dissolve 15 g potassium hexacyanoferrate(II) trihydrate ( $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ ) in water. Dilute to 100 ml with water.

**5.5 Magnesium acetate solution**

Dissolve 35,4 g of magnesium acetate  $[\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}]$  in 25 ml of water. Warm slightly to dissolve the magnesium acetate completely. Dilute to 100 ml with water.

**5.6 Litmus paper**

## 6 Apparatus

**6.1 Balance**, capable of weighing to the nearest 0,1 g.

**6.2 Water baths**, capable of operating at  $37\text{ °C} \pm 1\text{ °C}$ , at  $63\text{ °C} \pm 1\text{ °C}$ , and of boiling.

**6.3 Spectrometer**, suitable for measuring absorbance at a wavelength of 405 nm, or (optional) **special comparator**, with disc containing standard colour glasses, intended for visual comparison under reflected light and calibrated in micrograms of *p*-nitrophenol per millilitre of product, with two 25 mm diameter cells.

**6.4 Test tubes**, of diameter 16 mm, of length 150 mm, with matching stoppers.

After use, the test tubes should be cleaned by the following procedure: empty the tubes, rinse them in water, then wash in hot water containing an alkaline detergent and thoroughly rinse them again in clean hot water. Finally, rinse them in distilled water and dry before re-use. Rinse the test tube stoppers thoroughly in hot water after use, boil them in distilled water for 2 min and dry also before re-use. Alternative cleaning methods providing the same cleaning result may also be used.

**6.5 One-mark pipettes**, or **graduated pipettes**, of capacities 0,5 ml, 1 ml, 2 ml, 5 ml and 10 ml.

Thoroughly rinse the pipettes in clean water immediately after use, then rinse in distilled water and dry before re-use.

**6.6 Filter paper**, medium grade, of convenient size.

**6.7 Incubator**, capable of operating at  $34\text{ °C} \pm 1\text{ °C}$ .

## 7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 707.

## 8 Preparation of test sample and control sample

### 8.1 Milk, buttermilk and whey

Preferably, carry out the analysis directly after sampling. Otherwise, keep the test sample in a refrigerator (to avoid reactivation of inactivated enzyme) for no more than two days.

Mix the test sample carefully, if necessary with moderate heating, but under no circumstances allow the temperature to exceed  $35\text{ °C}$ .

### 8.2 Dried milk, buttermilk powder and whey powder

Weigh, to the nearest 0,1 g, 10 g of test sample. Dissolve it in 90 ml of water. Mix the thus-reconstituted test sample carefully by moderate heating, but under no circumstances allow the temperature to exceed  $35\text{ °C}$ .

### 8.3 Sour products

Add sufficient quantity of sodium hydroxide solution (5.1) to a sour or reconstituted sour product until a drop on litmus paper (5.6) just reacts as neutral.

## 8.4 Control sample

Boil a few millilitres of test sample (8.1, 8.2 or 8.3) carefully in a test tube (6.4) for more than 2 s. Ensure that the whole contents of the tube are well heated. Cool to room temperature.

## 9 Procedures

**WARNING — Avoid the influence of direct sunlight during the determination. Avoid any contamination with traces of saliva or perspiration which can give false positive results. In this respect, give special attention when pipetting.**

### 9.1 Procedure A (without clarification treatment and especially suitable for milk)

#### 9.1.1 Test portion

##### 9.1.1.1 Test solution

Pipette 5 ml of buffer substrate solution (5.3) into a clean dry test tube (6.4). Add 1 ml of prepared test sample (8.1, 8.2 or 8.3). Stopper the test tube and mix its contents by inversion.

##### 9.1.1.2 Control solution

Pipette 5 ml of buffer substrate solution (5.3) into an another clean dry test tube (6.4). Add 1 ml of control sample (8.4). Stopper the test tube and mix its contents by inversion.

#### 9.1.2 Determination

**9.1.2.1** Place both test tubes (9.1.1.1 and 9.1.1.2) for 2 h in the water bath (6.2) set at 37 °C.

**9.1.2.2** After 2 h, remove both tubes from the water bath. Mix each again by inversion. Remove the stoppers. Compare the test solution with the control solution, either visually or, if desired, using the special comparator (6.3).

#### 9.1.3 Test result

Consider the phosphatase reaction to be negative when the colour of the test solution is the same or visually nearly the same as that of the control solution in the comparison (9.1.2.2). Consider it to be positive when the colour of the test solution is distinctly more yellow.

In case of doubt, carry out a determination according to procedure B (9.2) or according to ISO 3356.

When using the special comparator (6.3), the *p*-nitrophenol content of the test solution is read with reference to the control solution, the tube containing the latter being positioned above the standard colour glasses in the disc. If artificial light is needed when taking these readings, the illumination should be as similar as possible to daylight.

If the reaction is considered to be positive, proceed according to 9.3.

## 9.2 Procedure B (with clarification treatment)

### 9.2.1 Test portion

#### 9.2.1.1 Test solution

Pipette 15 ml of buffer substrate solution (5.3) into a clean dry test tube (6.4). Add 2 ml of prepared test sample (8.1, 8.2 or 8.3). Stopper the test tube and mix its contents by inversion.

#### 9.2.1.2 Control solution

Again pipette 15 ml of buffer substrate solution (5.3) into another clean dry test tube (6.4). Add 2 ml of control sample (8.4). Stopper the test tube and mix its contents by inversion.

### 9.2.2 Determination

9.2.2.1 Place both test tubes (9.2.1.1 and 9.2.1.2) for 2 h in the water bath (6.2) set at 37 °C.

9.2.2.2 After 2 h, remove the tubes from the water bath. Remove the stoppers. Add 0,5 ml of zinc sulfate solution (5.4.1) to the contents of both tubes. Replace the stoppers and shake vigorously. Allow both tubes to stand for 3 min.

Remove the stoppers again. Add 0,5 ml of potassium hexacyanoferrate(II) solution (5.4.2) to the contents of both tubes. Mix thoroughly and filter the contents of each tube through fluted filter paper (6.6), collecting each filtrate in a separate dry clean test tube (6.4).

9.2.2.3 Compare the colour of the test solution with that of the control solution, either visually or, if desired, using the special comparator or spectrometer (6.3), the latter being set at 405 nm.

### 9.2.3 Test result

9.2.3.1 When comparing visually, consider the phosphatase reaction negative when the colour of the test solution is the same or nearly the same as that of the control solution, and positive when the colour of the test solution is distinctly more yellow. Use a spectrometer in case of doubt.

9.2.3.2 If the reaction is considered positive, proceed according to 9.3.

## 9.3 Tests for heat-stable and reactivated phosphatase

### 9.3.1 General

To ensure that a positive phosphatase test result obtained using either procedure A (9.1) or procedure B (9.2) is not due to the presence of heat-stable microbial phosphatase or the presence of reactivated phosphatase, carry out the following tests.

### 9.3.2 Test for heat-stable microbial phosphatase

#### 9.3.2.1 Sample pasteurization

Transfer 10 ml of the prepared test sample (8.1, 8.2 or 8.3) to two clean dry test tubes (6.4). Stopper one and place a thermometer in the other (control) tube to monitor the temperature.

Place both tubes in a water bath (6.2) set at 63 °C, so that the sample level is below the water level of the water bath. The temperature of the sample shall reach 63 °C within 5 min. Maintain the tubes at 63 °C for a further 30 min.

Remove the tubes from the water bath. Immediately cool them rapidly to room temperature in iced water while using the control tube to monitor the temperature.

### **9.3.2.2 Determination**

Examine the test sample according to the procedure (A or B) used with the test portion.

### **9.3.2.3 Test result**

If the laboratory-pasteurized test portion gives a positive phosphatase-activity result, then the test sample contains heat-stable phosphatase. Consider this result when using the method as a check for correct pasteurization of the product.

## **9.3.3 Test for reactivated phosphatase**

### **9.3.3.1 Preparation of test sample for dilution and control purposes**

Transfer 10 ml of the prepared sample (8.1, 8.2 or 8.3) to a clean dry test tube (6.4). Place the tube in a boiling-water bath (6.2). Check with a dry thermometer that the sample reaches 95 °C and keep it at this temperature for 1 min. Then cool rapidly to about room temperature in iced water. Use the thus-prepared sample for dilution (9.3.2.2) and as the (boiled) control.

### **9.3.3.2 Reactivation**

Transfer 5 ml of the prepared test sample (8.1, 8.2 or 8.3) to a clean dry test tube (6.4). Add 0,1 ml of water and mix (tube 1). Then transfer another 5 ml of the prepared test sample to an identical test tube (6.4). Add 0,1 ml of magnesium acetate solution (5.5) and mix (tube 2).

Incubate both tubes in the incubator (6.7) set at 34 °C for 1 h. Cool both rapidly to room temperature in iced water.

Transfer 1 ml of the contents of tube 2 to another dry clean new test tube (6.4). Dilute with 5 ml of the boiled test sample (9.3.2.1) and mix (tube 3). Do not dilute the contents of tube 1.

### **9.3.3.3 Determination**

Examine the contents of tube 1 and those of tube 3 according to procedure (A or B) used with the test portion.

### **9.3.3.4 Test results**

If the contents of tube 3 (diluted sample containing magnesium acetate) have equal or greater phosphatase activity than the undiluted sample in tube 1 (containing no magnesium acetate), then the test sample is considered negative for residual phosphatase.

This indicates that the phosphatase originally identified by procedure A (9.1) or B (9.2) is of reactivated origin.

If, however, the contents of tube 3 show less activity than those of tube 1, the test sample is considered positive for residual phosphatase [provided that the initial conventional phosphatase test according to procedure A (9.1) or B (9.2) was positive].

**NOTE** The original test may give a false positive result when a sample containing reactivated phosphatase is allowed to stand at elevated temperatures (21 °C to 24 °C) for more than 2 h.



## 10 Expression of results

Express the results as negative or positive, and/or “to be re-examined according to ISO 3356”.

When the colours are measured with a spectrometer or comparator (6.3), record the direct readings obtained in 9.1.2.3 or 9.2.2.3 as micrograms of *p*-nitrophenol per millilitre of sample or reconstituted sample, as appropriate.

Record readings between two standard colour glasses by adding a plus (+) or minus (–) sign to the value indicated by the nearest standard colour glass.

## 11 Precision

### 11.1 Interlaboratory test

The values for the repeatability were derived from the results of laboratory tests partly carried out in accordance with ISO 5725-1.

The values derived from this test may not be applicable to concentration ranges and matrices other than those given. National legislation or regulations may prescribe specific limits for the phosphatase activity of various products.

### 11.2 Repeatability

The absolute differences between two individual 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 2 µg of *p*-nitrophenol over the critical range from (0 to 14) µg of *p*-nitrophenol per millilitre of sample or reconstituted sample.

NOTE A comparator was used to obtain the above result which is more for guidance rather than a limit. The real precision figures are determined using the quantitative method specified in ISO 3356.

## 12 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 test method used, with reference to this Technical Specification;
- d) all operational details not specified in this Technical Specification, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained.

## **Annex A** (informative)

### **Alternative procedure using a patented proprietary reagent**

#### **A.1 Introduction**

A proprietary reagent is available for the rapid dissolution of colloidal milk components. This technique stops the enzymatic reaction, making it possible to obtain a perfectly clear mixture within a short time. The procedure specified in this annex is then applicable.

#### **A.2 Dissolving reagent**

Dissolving reagent is available commercially.

#### **A.3 Preparation of test sample and control sample**

See Clause 8.

#### **A.4 Procedure**

##### **A.4.1 Test portion**

See 9.2.1.

##### **A.4.2 Determination**

**A.3.2.1** Stopper the tubes and mix by inversion. Place the tubes in the water bath (6.2) set at 37 °C for 2 h.

**A.3.2.2** Remove the tubes from the water bath and treat each as follows.

Remove the stoppers. Add 8 ml of the dissolving reagent (A.2). Replace the stoppers and shake the tubes to mix their contents. Place the tubes in the water bath (6.2) set at 37 °C for 2 min.

**A.3.2.3** Compare the colour of the test solution with that of the control solution either visually or, if desired, with the use of a special comparator (6.3) or using a spectrometer (6.3) set at 405 nm.

**A.3.2.4** When comparing visually, consider the phosphatase reaction to be negative when the colour of the test solution is the same or nearly the same as that of the control solution, and positive when the colour of the test solution is distinctly more yellow. Use a spectrometer (6.3) in case of doubt.

## Bibliography

- [1] ISO 707, *Milk and milk products — Guidance on sampling*<sup>2)</sup>
- [2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*
- [3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*
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2) Equivalent to IDF 50.

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