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**Microbiology of food and animal feeding  
stuffs — Preparation of test samples,  
initial suspension and decimal dilutions  
for microbiological examination —**

**Part 4:  
Specific rules for the preparation of  
products other than milk and milk  
products, meat and meat products, and  
fish and fishery products**

*Microbiologie des aliments — Préparation des échantillons, de la  
suspension mère et des dilutions décimales en vue de l'examen  
microbiologique —*

*Partie 4: Règles spécifiques pour la préparation de produits autres que  
les produits laitiers, les produits carnés et les produits de la pêche*

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## ISO/CEN PARALLEL PROCESSING

The CEN Secretary-General has advised the ISO Secretary-General that this final draft International Standard covers a subject of interest to European standardization. Consultation on the ISO/DIS had the same effect for CEN members as a CEN enquiry on a draft European Standard. In accordance with the ISO-lead mode of collaboration as defined in the Vienna Agreement, this final draft, established on the basis of comments received, is hereby submitted to a parallel two-month FDIS vote in ISO and formal vote in CEN.

**Positive votes shall not be accompanied by comments.**

**Negative votes shall be accompanied by the relevant technical reasons.**

Documento de trabajo

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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.

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

ISO 6887-4 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 6887 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*:

- *Part 1: General rules for the preparation of the initial suspension and decimal dilutions*
- *Part 2: Specific rules for the preparation of meat and meat products*
- *Part 3: Specific rules for the preparation of fishery products*
- *Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, fish and fishery products*

Documento de trabajo

# Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination —

## Part 4:

### Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products

**WARNING** — The use of this standard may involve hazardous materials, operations and equipment. It is the responsibility of the user of this standard to establish appropriate safety and health practices and to determine the applicability of regulatory limitations prior the use.

## 1 Scope

This part of ISO 6887 specifies rules for the preparation of samples and decimal dilutions for the microbiological examination of food products other than those covered in other parts of ISO 6887. ISO 6887-1 defines the general rules for the preparation of the initial suspension and decimal dilutions for microbiological examination.

This part of ISO 6887 only describes methods of preparation that are applicable to several microorganisms simultaneously. It excludes the preparations that only apply to the detection and/or enumeration of a single microorganism where the methods of preparation are described in the relevant International Standard concerning that microorganism.

This part of ISO 6887 is applicable to the following products:

- general case for acidic products (see 8.2);
- foods with a high fat content, excluding margarine and spreads (see 8.3);
- flours, whole cereal grains, cereal by-products, animal feeds and cattle cake (see 9.1);
- very hard products, e.g. cassava (see 9.2);
- gelatine (see 9.3);
- margarine and spreads (see 9.4);
- dehydrated products and freeze-dried products (except dairy products and egg products) (see 9.5);
- egg and egg products (see 9.6);
- fermented products (products containing live microorganisms) (see 9.7);
- pastries and cakes (9.8).

NOTE Milk and milk products are dealt with in ISO 8261.

## 2 Normative references

The following referenced documents are 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 6887-1:1999, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions.*

ISO 6887-2:2003, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

## 3 Terms and definitions

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

### 3.1 laboratory sample

sample prepared for sending to the laboratory and intended for inspection or testing

[ISO 7002]

### 3.2 test portion

measured (volume or mass) representative sample taken from the laboratory sample for use in the preparation of the initial suspension

### 3.3 initial suspension (primary dilution)

suspension, solution or emulsion obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed with, normally, a nine-fold quantity of diluent, allowing large particles, if present, to settle

### 3.4 further decimal dilutions

suspensions or solutions obtained by mixing a measured volume of the initial suspension (3.3) with a nine-fold volume of diluent and by repeating this operation with further dilutions, until a decimal dilution series, suitable for the inoculation of culture media, is obtained

## 4 Principle

An initial suspension (3.3) is prepared to obtain as uniform a distribution as possible of the microorganisms contained in the test sample.

A pre-enrichment or enrichment suspension is prepared in the same way, using the medium recommended by the method of analysis concerned, except in special cases mentioned in each product section of this part of ISO 887.

If necessary, decimal dilutions (3.4) are prepared in order to reduce the number of microorganisms per unit volume to allow, after incubation, observation of any growth (in the case of liquid media) or colonies (in the case of agar plates or agar tubes), as stated in each specific standard.

In order to restrict, if required, the range of enumeration to a given interval, or if high numbers of microorganisms are foreseen, it is possible to inoculate only the necessary decimal dilutions (at least two successive dilutions) needed to achieve the enumeration according to the calculation described in ISO 7218.

## 5 Diluents

### 5.1 Basic materials

See ISO 6887-1.

### 5.2 Diluents for general use

#### 5.2.1 Peptone salt solution

See ISO 6887-1:1999, 5.2.1.

#### 5.2.2 Buffered peptone water

See ISO 6887-1:1999, 5.2.2.

### 5.3 Diluents for special purposes

#### 5.3.1 Peptone salt solution with Bromocresol purple

##### 5.3.1.1 Composition

Peptone salt solution (5.2.1)	1 000 ml
Bromocresol purple (0,04 % alcohol solution, e.g. ethanol solution)	0,1 ml

##### 5.3.1.2 Preparation

Add 0,1 ml of Bromocresol purple to 1 000 ml of peptone salt solution (5.2.1).

##### 5.3.1.3 Application

This solution may be used for the analysis of certain acidic products so that adjustment of the pH can be carried out without the use of a sterile pH probe (see 8.2).

Bromocresol purple is yellow at acidic pH, changing to purple at pH above 6,8.

#### 5.3.2 Phosphate buffered solution

##### 5.3.2.1 Composition

Disodium hydrogen phosphate dodecahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ )	9,0 g
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	1,5 g
Water	1 000 ml

##### 5.3.2.2 Preparation

Dissolve the components in the water, by heating if necessary.

If necessary, adjust the pH so that, after sterilization, it is  $7,0 \pm 0,2$  at 25 °C.

Place 180 ml into each flask.

Sterilize for 15 min in the autoclave set at 121 °C.

### 5.3.2.3 Application

Phosphate buffered solution is used as a diluent for gelatine (see 9.6) and other samples.

## 5.4 Distribution and sterilization of the diluent

See ISO 6887-1:1999, 5.4.

## 6 Apparatus

Usual microbiological laboratory equipment for general use (see ISO 7218 and ISO 6887-1) and, in particular, the following.

### 6.1 Homogenizer

#### 6.1.1 Rotary homogenizer (blender)

See ISO 7218. If a large test sample is used, the equipment should include a sterile 1 litre bowl.

#### 6.1.2 Peristaltic homogenizer

See ISO 7218.

### 6.2 Domestic-type grater, sterile.

### 6.3 Hammer

### 6.4 Water baths, capable of being maintained at $45 \pm 1$ °C, or $40 \pm 1$ °C, or between 37 °C and 42 °C.

### 6.5 Sterile scissors, knives, scalpels and forceps

### 6.6 Sterile spatulas, spoons or scoops

### 6.7 Sterile corers (metallic probes), for taking samples at depth.

### 6.8 Stirring apparatus, with to-and-fro motion.

### 6.9 Horizontal shaking apparatus

### 6.10 Wide-necked flasks, sterile, of capacity 500 ml.

## 7 Preparation of samples

### 7.1 Frozen products

Products stored frozen should be brought to a consistency that allows sampling; i.e. storing at 18 °C to 27 °C (laboratory temperature) for a maximum of 3 h, or  $2 \text{ °C} \pm 2 \text{ °C}$  for a maximum of 24 h. Samples should be tested as quickly as possible after that. See ISO 6887-1:1999, 9.3.

If the product is still frozen when portioning, some diluent at laboratory temperature may be used to facilitate defrosting.

Powders should be well mixed in their containers before sampling.

## 7.2 Hard and dry products

For hard or dry products, do not homogenize in a rotary homogenizer (6.1.1) for more than 2,5 min at a time.

For dry and hard or heterogeneous products, it may be necessary to mince or to grind the laboratory sample. In this case, to avoid an excessive rise in temperature, do not mince or grind for more than 1 min.

## 7.3 Liquid and non-viscous products

Before analysing, the test sample should be taken after having shaken the laboratory sample by hand (e.g. 25 times through an arc of 25 cm; see ISO 8261 for details) or by mechanical means in order to ensure that the microorganisms are uniformly distributed.

## 7.4 Heterogeneous products

For heterogeneous products (which contain pieces of different foods), sampling should be carried out by taking aliquots of each component representative of their proportions in the initial product.

It is also possible to homogenize the whole laboratory sample to allow the sampling of an homogenized test sample.

It may be necessary to mince or to grind the laboratory sample. In this case, to avoid an excessive rise in temperature, do not mince or grind for more than 1 min.

# 8 General procedures

## 8.1 General

All preparations and manipulations should be carried out using good aseptic techniques and with sterile equipment to prevent microbial contamination of samples from all external sources. See ISO 7218.

Indicate in the report which procedure is used for analysis if it is different from the procedure described in this part of ISO 6887.

## 8.2 General case for acidic products

It is important when using a suspension solution of acidic products to ensure that the pH is brought back to neutral. The use of diluent with an added pH indicator (5.3.1) can avoid the need to use and sterilize pH probes: add sodium hydroxide (NaOH) to bring back the coloration of the suspension until the indicator starts to change.

For use with buffered diluents, the addition of NaOH is often necessary to increase the buffering capacity of the alkaline component. The concentration of added NaOH is depends on the product acidity. The most suitable concentration (e.g. 0,1 mol/l or 1 mol/l) is the concentration which is still close to a ratio of 1 in 9 with diluent.

## 8.3 High fat foods, excluding margarines and spreads (e.g. over 20 % of total mass is fat)

The use of a diluent with between 1 g/l and 10 g/l of added sorbitan monooleate (Tween 80), approximately according to fat levels (e.g. at a fat content of 40 %, add 4 g/l) may improve emulsification during suspension.

## 9 Specific procedures

### 9.1 Flours, cereal grains, cereal by-products, animal feeds and cattle cake

#### 9.1.1 General

Follow sampling plans according to the size of each batch of product.

#### 9.1.2 Preparation of initial suspension

Mix dry powders well by hand in their container before weighing out the test sample.

Add 1 part test sample to 9 parts of peptone salt solution (5.2.2) and mix.

Before homogenization, leave to stand for 20 min to 30 min at 18 °C to 27 °C (laboratory temperature).

If the viscosity of the suspension increases so that it becomes too thick or viscous to mix well or pipette, add a further equal volume of peptone salt solution to produce a 1 in 20 initial suspension.

Mix according to the product with either a peristaltic homogenizer (6.1.2) for 1 min, or in a rotary blender (6.1.1).

In the enumeration calculation, take into account any additional dilutions made.

A test portion of 50 g is recommended when analysing cereals and other heterogeneous products. In this case, the first suspension should be a 1 to 5 suspension. Homogenize and make a 1 in 2 dilution.

NOTE Hard materials (e.g. grains and bone meal) will puncture bags for a peristaltic homogenizer; double bagging may prevent this.

### 9.2 Very hard products (e.g. cassava)

#### 9.2.1 Preparation of test sample

Take a larger amount of the test sample than is required for analysis and aseptically grate (6.2) into small pieces or break into small pieces in a sterile plastic bag using a hammer (6.3).

#### 9.2.2 Preparation of initial solution

Add 1 part test sample to 9 parts of peptone salt solution (5.2.1) and mix.

Before homogenization, leave to stand for 20 min to 30 min at 18 °C to 27 °C (laboratory temperature).

Blend in a rotary blender (6.1).

### 9.3 Gelatine (granular or leaf form)

#### 9.3.1 Preparation of test sample

Remove 20 g of the test sample using an aseptic technique.

#### 9.3.2 Preparation of initial solution

Transfer the test sample to a 500 ml sterile flask (6.10). Add 180 ml of phosphate buffered diluent (5.3.2) and mix to disperse the granules in the liquid.

Leave the gelatine to adsorb the diluent for 60 min at room temperature.

Place the flask in a water bath (6.4) set at 45 °C for a maximum of 30 min and mix frequently to dissolve the gelatine and give a 1 + 9 solution.

## 9.4 Margarine and spreads

### 9.4.1 Sampling

#### 9.4.1.1 General

Samples may be taken from within the bulk product or from within and/or on the surface of packeted ready for sale items.

#### 9.4.1.2 Bulk or pre-wrapped product of > 1 kg

Using aseptic methods, use a spatula (6.6) or a knife (6.5), to remove a slice 3 mm to 5 mm thick from the outer layer.

Push a sterile metallic corer (6.7) into the product diagonally without going all the way through. Turn the corer in a full circle then remove the conical test sample.

Transfer the sample to a sterile container or plastic bag, using the spatula (6.6) or knife (6.5), apart from the upper 25 mm which is used to block the hole made by the corer (6.7).

Take one or more core samples to obtain an adequate test sample for laboratory use.

Any other sampling method (such as extracting a mass of at least 500 g) is permitted so long as the product is regarded as homogeneous. This shall, however, be stated in the test report.

#### 9.4.1.3 Pre-wrapped margarine product of $\leq 1$ kg

The laboratory sample used is made up of one or several pre-wrapped, intact items.

Take samples using aseptic methods. If the laboratory sample is of a mass greater than 500 g of bulk product, the test sample shall be taken after the outer layer has been removed to a depth of 5 mm.

#### 9.4.1.4 Pre-wrapped products

For pre-wrapped products, open the packaging. Take the sample using a sterile instrument, after the outer layer has been removed. It may also be taken using a corer to take a cylindrical portion through the sample.

If requested, a sample may also be removed from the product surface using a sterile instrument.

### 9.4.2 Preparation of test sample

#### 9.4.2.1 General

In a sterilized, calibrated flask, weigh out 40 g from the laboratory sample.

#### 9.4.2.2 Preparation of the aqueous phase (primary dilution)

Add a volume of diluent (5.2) to a sterile container, equivalent to the expected fat proportion of the margarine or spread sample.

EXAMPLE For margarine with 82 % fat and a 40 g test sample, add  $40 \times 0,82 = 33$  ml of diluent.

Place the container in the water bath (6.4) set at 45 °C until the product has completely melted. This time should not exceed 20 min.

Mix using a shaker with the stirring apparatus (6.8) until a homogeneous emulsion is produced. The mixing time varies from 2 min to 5 min depending upon the type of margarine/spread.

Leave the container at ambient temperature so that the fatty (upper) layer and the aqueous (lower) are properly separated.

Carry out further operations on the aqueous layer; 1 ml of this solution corresponds to 1 g of margarine. This is the sample to be used to prepare the initial suspension in accordance with ISO 6887-1.

#### 9.4.2.3 Preparation of an enrichment or pre-enrichment suspension

See the relevant International Standard for the microorganism to be detected or enumerated.

If the method requires enrichment or pre-enrichment, the sample may be a complete portion of margarine.

### 9.5 Dehydrated products

#### 9.5.1 General

The following are regarded as dehydrated products:

- dehydrated meats and vegetables; dry soups and gravy mixes;
- powdered drinks: tea, cocoa and chocolate-based products, coffee, dehydrated fruit juice (powdered);
- raw cellulose, soluble cellulose, dextrines, sorbitol, sugars, glucose, glutamate;
- herbs, spices, flavourings and colourings;
- polysaccharide gelling agents, gums, etc.;
- coconut, yeast extract, chocolate confectionery (bars or sweets), dehydrated whole egg and dried white of egg.

The following are excluded:

- milk products;
- egg products;
- live microorganisms (e.g. yeasts for bakery).

#### 9.5.2 Apparatus

Peristaltic homogenizing bags with filter tube inserts are recommended to assist in the pipetting of products with substantial insoluble matter in suspension.

#### 9.5.3 Preparation of test sample

Powdered products should be thoroughly mixed in their container then weighed out directly using aseptic methods. Other products may need to be broken up or cut with sterile tools into small pieces prior to use.

## 9.5.4 Preparation of initial suspension

### 9.5.4.1 Powdered products, completely soluble

As these products are soluble, it is not always necessary to carry out a mechanical homogenization.

### 9.5.4.2 Other (non-powdered) products

Prepare a suspension using a rotary blender (6.1.1) or peristaltic homogenizer (6.1.2), as above.

### 9.5.4.3 Products which swell in water

For all products which swell in water (e.g. polysaccharides and gum gelling agents, dehydrated parsley or chives), make further dilutions with diluent (1 in 20, 1 in 50 or 1 in 100) to gain a usable suspension.

Where greater dilutions are made, the number of inoculated plates shall be increased in order to distribute 0,1 g of the test sample when low counts are expected.

The solubility of some substance can be aided by the addition of a specific enzyme solution to the buffered peptone water (5.2.2) (e.g. gamanase for carob and guar-based products, or cellulase for carboxymethyl cellulose).

### 9.5.4.4 Additional dilutions for inhibitory food materials

For certain food additives which contain inhibitory substances (e.g. onion powder, garlic, oregano, peppers and certain teas and coffee), in order to decrease the antimicrobial activity

- use greater dilutions (e.g. 1/100 for cinnamon and oregano, and 1/1000 for cloves), or
- add  $K_2SO_4$  to the buffered peptone water (5.2.2) to achieve a final concentration of 0,5 %.

### 9.5.4.5 Chocolate and chocolate confectionery (bars or sweets)

Pre-heat the diluent to 40 °C.

Add the weighed test sample to the diluent. Mix immediately by hand.

Leave the mixture at room temperature for 20 min to 30 min to liquidize. Then mix using the peristaltic homogenizer (6.1.2).

## 9.5.5 Resuscitation

In general, leave the sample to rest for about 30 min  $\pm$  5 min at laboratory temperature. Do not exceed a temperature of 25 °C before preparation of further dilutions. Individual cases should be considered specifically.

## 9.6 Egg products

### 9.6.1 Fresh whole eggs

#### 9.6.1.1 General

Eggs used for microbiological analysis should not have any visible cracks in them. Eggs may be examined singly or in batches according to purpose.

Examination may be carried out with or without cleaning/sterilizing the eggshell. For examination of the contents, sterilize the eggs before opening. For the detection of pathogens which may also be on the outside of the shell, sterilization of the shell may not be required according to the agreement between parties.

#### 9.6.1.2 Disinfecting the shell

Remove any dirt or faeces with a tissue and water and blot dry.

Wearing sterile gloves and using a gauze or wipe soaked in either 70 % industrial methylated spirit or isopropanol, wipe the whole of the shell surface.

Iodine solution may also be used, with precautions to protect the personnel.

Allow to dry completely without contamination.

#### 9.6.2 Detecting or counting shell flora

##### 9.6.2.1 General

The egg shall always be handled using aseptic techniques.

##### 9.6.2.2 Rinsing the shell method

Without breaking the egg, rinse it several times, turning it around, using known volumes of small amounts of the diluent or medium used in the method.

The resulting liquid, which is collected in the container, becomes the initial suspension.

Also the whole intact egg may be placed in a peristaltic homogenizer bag that contains a known volume of the diluent or culture medium. Then, through the bag, rinse the egg in the liquid in the bag, then remove it.

##### 9.6.2.3 Friction method

Using sterile gauze, or any other equivalent fabric/paper soaked in diluent or the culture medium, rub the whole eggshell.

Place the piece of gauze in the volume of diluent (5.2.1) or culture medium required for the analysis.

##### 9.6.2.4 Soaking method

Break the egg.

Take the shell and place it in the homogenization bag with the required volume of diluent or culture medium.

Knead it in the bag and use the resulting suspension.

#### 9.6.3 Detecting or counting internal flora

Using fresh sterile gloves, break the egg open aseptically.

If the yolk and white are to be analysed separately, separate them and place each in a different sterile container.

Add peptone salt solution (5.2.1) to give a 1 + 9 dilution for the yolk and 1 in 40 for the white to dilute out the naturally occurring lysozyme inhibitor.

If detection of microorganisms is carried out on the whole egg contents, place the contents directly in a sterile container with 180 ml of buffered peptone water (5.2.2) or into the appropriate enrichment broth.

#### 9.6.4 Bulk whole liquid egg, bulk liquid whites egg and bulk liquid yolk

These may be pasteurized or not.

For bulk whole liquid egg, dilute 1 + 9 with buffered peptone water (5.2)

For egg whites, a 1 in 40 suspension in peptone water (5.2) is recommended to overcome the inhibition caused by naturally occurring lysozyme.

#### 9.6.5 Dehydrated whole egg and dried egg white

Treat as for dehydrated products as in 9.5.

#### 9.6.6 Counting all flora (shell + yolk + white)

Using aseptic techniques, break the egg and place the shell and its contents in a sterilized container.

Homogenize by hand shaking/crushing.

Take the required mass of the mixture to make the initial suspension in a diluent.

### 9.7 Fermented products (products containing live microorganisms)

#### 9.7.1 General

This clause excludes probiotic products.

The purpose is to examine products for contamination by microorganisms other than those used in the fermentation.

#### 9.7.2 Diluent

Use peptone salt solution with Bromocresol purple (5.3.1)

When the suspension indicates a change in colour of the indicator, add 40 g/l of sodium hydroxide to return to a near neutral pH (e.g.  $7,0 \pm 0,2$  at  $25^{\circ}\text{C}$ ).

In the case of yeast, an anti-fungal agent (e.g. cycloheximide at a concentration of 50 mg/kg, or nystatin at 50 mg/kg, or amphotericin at a concentration of 10 mg/kg) shall be added to the counting medium.

In other cases, it is recommended that an antibiotic be added to act against the flora contained in the product being analysed. This antibiotic and its concentration shall be mentioned in the test report.

### 9.8 Pastries and cakes

#### 9.8.1 General

Pastries and cakes are generally sweet and made from flour, butter, eggs and other ingredients such as dairy products or fruit products.

#### 9.8.2 Preparation of test sample

For packaged pre-cooked foods, open up the packaging in compliance with ISO 6887-2:2003, 9.2.

Take aliquots of each component, taking their proportions into account.

It is possible to homogenize the laboratory sample in its entirety in order to get a homogenized test sample.

It is recommended to treat biscuits in the same way as dehydrated products (9.5)

## 10 Further decimal dilutions

See ISO 6887-1.

Documento de trabalho

## Bibliography

- [1] ISO 7002, *Agricultural food products — Layout for a standard method of sampling from a lot*
- [2] ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

Documento de trabalho

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