Preparation of samples and dilutions, plating and sub-culture

National Infection Service
Food Water and Environmental Microbiology
Standard Method
About Public Health England

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Published January 2020
PHE publications gateway number: GW-980

PHE supports the UN Sustainable Development Goals
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Status of National Infection Service food, water and environmental microbiology methods

These methods are well referenced and represent a good minimum standard for food, water and environmental microbiology. However, in using Standard Methods, laboratories should take account of local requirements and it may be necessary to undertake additional investigations.

The performance of a standard method depends on the quality of reagents, equipment, commercial and in-house test procedures. Laboratories should ensure that these have been validated and shown to be fit for purpose. Internal and external quality assurance procedures should also be in place.

Whereas every care has been taken in the preparation of this publication, Public Health England (PHE) cannot be responsible for the accuracy of any statement or representation made or the consequences arising from the use of or alteration to any information contained in it. These procedures are intended solely as a general resource for practising professionals in the field, operating in the UK, and specialist advice should be obtained where necessary. If you make any changes to this publication, it must be made clear where changes have been made to the original document. PHE should at all times be acknowledged.

Citation for this document:
## Amendment history

<table>
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<tr>
<th>Page</th>
<th>Section(s) involved</th>
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</tr>
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<tbody>
<tr>
<td>All</td>
<td>All</td>
<td>Updated to reflect revised template FNEW10 version 7</td>
</tr>
<tr>
<td>8</td>
<td>1.0 Principle</td>
<td>Reference to ISO 7128 added (CR14358). Inclusion of time tolerance for ambient stable food (CR16085) and shelf-life testing.</td>
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<td>9</td>
<td>3.0 H&amp;S considerations</td>
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<td>Safety critical task added</td>
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<td>13</td>
<td>7.2 Preparation of initial suspension</td>
<td>±5% changed to +5% only (CR15771)</td>
</tr>
<tr>
<td>19</td>
<td>7.4 Plating of homogenates</td>
<td>Information note added to recommend plating from least to most selective media (CR15113)</td>
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<td>8.3 Solid to solid</td>
<td>Information note added to recommend plating from least to most selective media (CR15113)</td>
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<tr>
<td>18</td>
<td>Figure 1</td>
<td>Typo corrected (CR13572)</td>
</tr>
<tr>
<td>25</td>
<td>References</td>
<td>Updated (CR15710)</td>
</tr>
</tbody>
</table>

The amendments that have occurred since the previous version of this document are shown below. On issue of revised or new documents each controlled document should be updated by the copyholder in the laboratory.
Introduction

Scope

The procedures described are applicable to the microbiological examination of food and dairy samples.

Background

Public Health England (PHE) Food, Water and Environmental Microbiology Laboratories test food and environmental samples that are collected as part of food poisoning investigations, for national and local studies, for routine investigation of food premises and for the purpose of official control.

Examination of food and dairy samples for a range of micro-organisms is performed in order to meet statutory requirements and food safety guidance, to complete surveillance on the microbiological quality of food products and to investigate complaints and outbreaks. The following document describes the sample preparation procedures necessary for the detection and enumeration of organisms in food and dairy samples.

The procedures described are based on those detailed in EN ISO 6887 parts 1-4 and EN ISO 7218:2007+Amd 1:2013.

Differences between this method and EN ISO Standard 6887 and ISO 7218 and other horizontal methods are below:

<table>
<thead>
<tr>
<th>PHE method F2</th>
<th>EN ISO 6887</th>
<th>Justification for variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of initial suspension</td>
<td>For dehydrated products that absorb moisture, dilutions of 1 in 20, 1 in 50 or 1 in 100 may be used. Subsequent calculations of detection limits / dilution factors must then be adjusted accordingly.</td>
<td>Part 4: for products which swell in water, dilutions of 1 in 20, 1 in 50 or 1 in 100 may be used. The number of inoculated plates should then be increased to distribute 0.1g of sample in total when low counts are expected.</td>
</tr>
</tbody>
</table>
| Preparation of initial suspension | Acidic products are not covered separately. | Acidic products are described specifically in part 4. It is recommended that | The preparation of a 1 in 10 dilution, followed by further dilutions and/or inoculation of solid or liquid media would be
<table>
<thead>
<tr>
<th>Preparation of initial suspension</th>
<th>25g or 25mL of sample used for all product types</th>
<th>Part 5: 10g quantity is specified for several types of dairy product</th>
<th>In order to achieve consistency throughout the method, it is considered preferable to use 25g/25mL for all product types</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHE method F2</td>
<td>EN ISO 7218:2007 + Amd 1:2013</td>
<td>Justification for variation</td>
<td>The use of duplicate plates at each dilution to achieve a weighted mean is not considered essential where the focus is on identifying bacterial levels that pose a risk to public health. The impact of plating variation is addressed by determining method uncertainty. Official control samples that have been submitted strictly in accordance with sampling plans and formal samples are tested in duplicate and weighted mean counts determined because the methodology used in these circumstances is liable to challenge in a court of law.</td>
</tr>
<tr>
<td>Plating of homogenates</td>
<td>Plating of 50uL, 0.1mL, 0.5mL and/or 1mL to single plates</td>
<td>Plating of 1mL over 3 plates and further serial dilutions in duplicate.</td>
<td></td>
</tr>
<tr>
<td>PHE method F2</td>
<td>Specific Horizontal Methods for detection of food pathogens</td>
<td>Justification for variation</td>
<td></td>
</tr>
<tr>
<td>Sub-culture of enrichment broths</td>
<td>Recommends use of 2 loops to achieve single colonies on a single plate.</td>
<td>Recommends sub-culture to 2 plates using the same loop.</td>
<td>Procedure described for PHE methods ensures well isolated colonies are obtained on a single plate.</td>
</tr>
</tbody>
</table>
1.0 Principle

An initial suspension of sample is prepared in such a way as to obtain as uniform a distribution as possible of the microorganisms contained in the test portion.

A pre-enrichment or enrichment suspension may also be prepared in the same way, using medium recommended by the specific standard method concerned.

If necessary, further decimal dilutions are prepared in order to reduce the number of microorganisms per unit volume to allow, after incubation, the observation of their growth (in tubes or bottles) or the counting of colonies (on plates) in samples that contain high numbers of organisms.

Routine samples are tested by inoculation of single plates and are not tested in duplicate or using dilution series as stated in ISO methods. The justification for the use of single plates for routine testing is that the priority for PHE testing is to detect levels of bacteria that indicate a risk to public health, rather than to achieve a high level of accuracy at low levels of contamination. As such, it is considered that a method that gives a detection limit of <20 Colony forming units per gram (CFU per g) (ie single 0.5mL plate at 10⁻¹ dilution) rather than <10 CFU/g (ie duplicate 0.5mL plates at 10⁻¹ dilution) is sufficient. Similarly, the use of duplicate plates at several dilutions to achieve a weighted mean is not considered essential where the focus is on identifying bacterial levels that pose a risk to public health. The impact of plating variation is addressed in each laboratory by determining method uncertainty using IQC and EQA data.

For official control samples that are submitted strictly in accordance with sampling plans and for formal samples testing in duplicate and determining weighted mean counts is performed because the methodology used in these circumstances is liable to challenge in a court of law.

For highly perishable products (e.g. shellfish, salad vegetables), testing should commence within 24 h of sampling. For perishable products (e.g. cooked meats, fish, raw milk) and ambient stable products, testing should commence within 36 h⁶. Testing food at the end of its shelf-life can also be done on customer request but only if the product has been suitably stored.
2.0 Definitions

Sample preparation
The steps involved in the preparation of the initial suspension of the sample.

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 an appropriate quantity of diluent.

Further decimal dilutions
Suspensions or solutions obtained by mixing a measured volume of the initial suspension 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.

3.0 Safety considerations

3.1 General safety considerations

Normal microbiology laboratory precautions apply. All laboratory activities associated with this SOP must be risk assessed to identify hazards. Appropriate controls must be in place to reduce the risk to staff or other groups. Staff must be trained to perform the activities described and must be provided with any personal protective equipment (PPE) specified in this method. Review of this method must also include a review of the associated risk assessment to ensure that controls are still appropriate and effective. Risk assessments are site specific and are managed within safety organiser.

Information Note: Throughout this method safety critical tasks are highlighted in yellow and identified using the exclamation mark symbol. Safety Critical tasks (or processes) are defined as “…one that if carried out incorrectly or not at all could lead to death, significant injury, ill health, loss of containment or serious plant/equipment damage”.

Hazards are identified using red text. Where a means of controlling a hazard has been identified this is shown in green text.
3.2 Specific safety considerations

Food products must be handled with appropriate care, depending on their inherent risks. For example, consideration should be given to the handling of common food allergens such as nuts in a way that avoids the creation of excessive dust and aerosols. Unpasteurised milk and raw meats have a higher likelihood of being contaminated with a range of pathogenic micro-organisms, and appropriate measures must be taken when handling these in the laboratory. The sub-sampling of certain hard or tough food and feed products (e.g. dried meat and animal hide dog chews), and the opening of containers such as tins, may require the use of sharp utensils. When using these utensils, wear protective gloves, ensure that the food item is held securely within a cut-proof container or tray before cutting, and use can-openers that are secured to the bench if available.

3.3 Laboratory containment

All procedures can be performed in a containment level 2 (CL2) laboratory, unless risk assessment of the product or circumstances of its submission (e.g. outbreak) suggest that the food or dairy item is likely to be contaminated with HG3 organisms (e.g. Salmonella Typhi and Paratyphi, E. coli O157 or STEC). In this case, the sample should be handled in a containment level 3 (CL3) Laboratory.

4.0 Equipment

- Top pan balance capable of weighing to 0.1g.
- Gravimetric diluter (optional)
- Stomacher
- Pulsifier (optional)
- Vortex mixer
- Stomacher bags (sterile) with mesh insert if necessary and wire closures.
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Fine tipped pipettes (spiral plater)
- Beakers (spiral plater)
- Waterbaths/incubators at 37 ± 1°C and 45 ± 1°C
- pH meter capable of measuring to 0.1 units
- Sterile spatulas/spoons/scoops
- Sterile scissors/knives/forceps (optional)
- Sterile tray (optional)
5.0 Culture media and reagents

Equivalent commercial dehydrated media may be used; follow the manufacturer’s instructions.

Peptone saline diluent (Maximum recovery diluent)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>8.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
<tr>
<td>pH 7.0 ± 0.2 at 25°C</td>
<td></td>
</tr>
</tbody>
</table>

Buffered peptone water (ISO formulation)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic digest of casein</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate dodecahydrate</td>
<td>9.0 g</td>
</tr>
<tr>
<td>or anhydrous disodium hydrogen phosphate</td>
<td>3.5 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
<tr>
<td>pH 7.0 ± 0.2 at 25°C</td>
<td></td>
</tr>
</tbody>
</table>

Information note: When preparing samples for Listeria enumeration testing BPW or ½ Fraser broth can be used refer to national method FNES22⁹.

Sodium citrate diluent

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisodium citrate dihydrate ((\text{Na}_3\text{C}_6\text{H}_5\text{O}_7.2\text{H}_2\text{O}))</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
<tr>
<td>pH 7.5 ± 0.2 at 25°C</td>
<td></td>
</tr>
</tbody>
</table>

Dipotassium hydrogen phosphate diluent

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipotassium hydrogen phosphate ((\text{K}_2\text{HPO}_4))</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
<tr>
<td>Final pH 7.5 ± 0.1 (for preparing homogenates of cheese, processed cheese, caseinates, and sour cream) or 8.4 ± 0.1 at 25°C (for acid casein powder, lactic casein powder and acid whey powder).</td>
<td></td>
</tr>
</tbody>
</table>
6.0 Aseptic technique

When handling samples or cultures, aseptic technique is essential to avoid contamination of the sample and to protect the laboratory worker from infection.

The following points must be observed when preparing samples or performing subcultures.

- Caps and lids from containers should not be placed on the workbench but retained in the hand whilst the sample is being processed. Caps and lids must be replaced as soon as possible.
- Keep samples away from the face when opening culture containers.
- Minimise the production of aerosols by opening caps slowly, after mixing allow universal to stand for a minute prior to opening.
- If forceps or scissors are used when handling samples these must be sterilised by autoclaving or decontaminated using 70% IMS or 2500 ppm hypochlorite prior to use. Safety eyewear and gloves must be worn.
- All consumable items used to prepare samples such as, loops, pipettes, pipette tips, hockey sticks, bags and containers must be sterile single use items.
- Ensure that samples do not come into contact with sterile equipment (eg metal ends of dilumat tubing). If this occurs, the equipment must be replaced to prevent cross contamination of further samples.
- When pouring liquid media ensure that the mouth of the bottle does not come into contact with samples or non-sterile items. If this occurs, discard the media.
- Samples should be prepared in such a way as to ensure that no bacterial contamination is introduced by contact with hands or contact with unsterile surfaces or items.
3.0 Sample processing

7.1 Sample preparation

**Homogeneous samples** including powders and free flowing liquids and concentrates should be mixed well before removing a portion for testing (for example, by shaking 25 times). Do not shake powders immediately before testing as the environment may become contaminated by dust particles.

**Heterogeneous samples** such as sandwiches should usually be sampled by removing a representative portion of the whole product so that all components are taken in similar proportions to that in the food.

**Products stored frozen** should be brought to a consistency that allows sampling (i.e. storing at 18-27°C for a maximum of 3 h or at 5°C ± 3°C for a maximum of 24 h). Samples should be tested as soon as possible after that. Store thawing samples on separate trays to prevent cross-contamination from any drips leaking through packaging.

**Packaged products** should be opened aseptically, and if necessary and wearing safety glasses and gloves (if the package cannot be opened without risk of external contamination), the external surface of the package should be disinfected by wiping with alcohol prior to opening or with 2500ppm of hypochlorite solution if spore forming bacteria are being sought in the sample.

All diluents should be pre-warmed to room temperature prior to use. Homogenates of most types of food and dairy product may be prepared using a stomacher or pulsifier.

Use aseptic techniques throughout all sampling and handling procedures to avoid the introduction of extraneous micro-organisms. Containers, stomacher bags and any utensils used for sampling must be sterile.

**Pooling of samples** can be performed when more than one 25g test portion from a specified product batch is to be examined and when evidence is available that combining test portions does not affect the result for that particular food¹. This can either be done by combining 25g of each sub-sample into a larger volume, and then preparing an initial suspension using the entire combined volume of sample, or (for enrichment tests) by preparing separate enrichment broths for each sub-sample and then combining a portion of each broth for subsequent testing.
7.2 Preparation of initial suspension

As soon as practicable after sample receipt or as agreed with the customer (e.g. end of shelf life testing), using sterile containers, instruments and using aseptic technique, weigh a representative 25 g sample (to a tolerance of ± 5% i.e. ±1.25g ) of each food into a sterile stomacher bag. Record the weight. Smaller amounts may be examined without additional validation or verification provided the same ratio of enrichment broth to test portion is maintained. This is more likely to be required for low density products such as herbs, when testing certain product for legislative compliance or when specified by the customer. Avoid touching the inside of the bag with the hands. It is advisable to use a stomacher bag containing a mesh insert for samples high in fat or fibre to retain particles that can interfere with pipetting.

Information note: Where the TEMPO automated technique is used for subsequent enumeration tests, a stomacher bag with fine mesh must be used.

Add exactly 9 times the weight or volume of peptone saline diluent (PSD) (to a tolerance of ±2% i.e if sample weight is 25g exactly, final weight would be 250g ± 5 g) at ambient temperature to give a 1 in 10 (10⁻¹) suspension.

A single buffered peptone water (BPW) homogenate may be used for both detection of Salmonella and enumeration of other organisms. In this instance prepare the homogenate by weighing at least 27 g of sample, add an appropriate volume of BPW, and aseptically remove 20 mL of this 10⁻¹ homogenate to a sterile universal for enumeration and use the remainder of the homogenate for detection of Salmonella.

Information note: Section 7.2.1 –7.2.15 list exceptions based on different food types.

Record the weight or volume used. If the amount of food available is less than 25 g, maintain the sample: diluent ratio at 1:9 (1 in 10). Preparation of 1 in 20, 1 in 50 or 1 in 100 homogenates may be necessary for some dehydrated products such as dried herbs which absorb moisture, or products with a high starch content which might produce a homogenate of too great a viscosity to handle. Greater dilutions may also be used for food ingredients that contain inhibitory substances (e.g onion powder, garlic, oregano, cinnamon, cloves, peppers and certain teas and coffees).

Information note: Changes in the concentration of homogenate must be taken into account in subsequent calculations.

Using a stomacher, homogenise the suspension for 30 seconds to 3 minutes depending on the nature of the product and type of stomacher in use. Samples
which may puncture the stomacher bag must be placed inside a further 2 or 3 stomacher bags to prevent leakage or alternatively, a pulsifier may be used for the homogenisation process to avoid direct pressure on the bag. Manual homogenisation is also permissible, samples must be manually homogenised for a minimum of 2 minutes.

Allow large particles to settle, if necessary, for up to 15 minutes, if stomacher bags with mesh have not been used. In general, leave dehydrated products to rest for about 30 min at room temperature before preparation of further dilutions, in order to allow resuscitation of micro-organisms to take place.

Specific requirements for the preparation of particular products are described below and summarised in Table 1.

7.2.1 Milk and liquid milk products

Rapidly invert the sample container 25 times. Avoid foaming or allow any foaming to disperse before sampling. Within 3 minutes of mixing, remove 25 mL of sample and add 225 mL peptone saline diluent. Shake thoroughly eg 25 times in 7 seconds.

7.2.2 Cheese and processed cheese

Weigh 25 g into a stomacher bag. Add 225 mL of sodium citrate solution or dipotassium hydrogen phosphate solution pH 7.5. Blend in a stomacher until the product is thoroughly dispersed (30 seconds to 3 minutes).

7.2.3 Frozen milk products (including edible ices containing milk products)

Weigh 25 g of the test sample into a container. Add 225 mL of peptone saline diluent at room temperature. Mix in a stomacher until thoroughly dispersed (1 minute).

7.2.4 Butter

Weigh 25 g of the test sample into a container. Place the container in a waterbath at 45 ± 1°C until the whole test portion has just melted. Add 225 mL of peptone saline diluent pre-warmed to about 45°C. Mix in the stomacher until thoroughly dispersed (1 minute).
7.2.5 Fermented milk, yoghurt and sour cream

Weigh 25 g of the test sample into a container. Add 225 mL of dipotassium hydrogen phosphate solution pH 7.5 and mix in a stomacher (1 minute).

7.2.6 Dried milk, dried sweet whey, dried acid whey, dried buttermilk and lactose

Use dipotassium hydrogen phosphate solution, pH 7.5, for roller-dried milk. Use peptone saline solution for dried sweet whey, buttermilk and lactose. Use dipotassium hydrogen phosphate solution, pH 8.4, for dried acid whey. Thoroughly mix the contents of the closed sample container by repeatedly shaking and inverting. If the container is too full to allow thorough mixing, transfer the contents to a larger container and mix.

Remove 25 g of sample and add to a bottle containing 225 mL of the diluent. In order to dissolve, swirl slowly to wet the powder, then shake the bottle 25 times in about 7 seconds or mix in a stomacher. Allow to stand for 5 minutes, shaking occasionally.

The diluent may be warmed to 45°C if a homogeneous suspension cannot be obtained even after blending. Ensure a record is made of this additional procedure if it is used.

7.2.7 Acid casein, lactic casein, rennet casein and caseinate

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it.

Weigh 25 g of sample into a sterile plastic stomacher bag and add 225 mL of diluent as follows:
- Acid and lactic casein: dipotassium hydrogen phosphate solution, pH 8.4
- Caseinate: dipotassium hydrogen phosphate solution, pH 7.5
- Rennet casein: dipotassium hydrogen phosphate solution, pH 7.5

Mix well manually and allow to stand at room temperature for 15 min. Blend if necessary for 2 min in a stomacher, using double bags for granular products. Allow to stand for 5 min before preparing further dilutions.
7.2.8 Chocolate and chocolate confectionery

Pre-heat peptone saline diluent to 40°C. Add the weighed test sample to the diluent. Mix immediately by hand. Leave the mixture at room temperature for 20 – 30 min to liquidize, then mix in a stomacher.

7.2.9 Hard and dry products

Do not homogenize hard or dry products in a rotary homogenizer for more than 2.5 min at one time to avoid an excessive rise in temperature.

7.2.10 Liquid and non-viscous products

Before taking the test portion, the laboratory sample should be shaken by hand (eg 25 times through an arc of 25 cm) in order to ensure that the microorganisms are uniformly distributed.

7.2.11 Acidic products

It is important when preparing a suspension of acidic products that the pH is brought back to near neutrality (pH 7.0 ± 0.5). The use of buffered peptone water is sufficient for most products with pH greater than or equal to 4.5. More acidic products (greater than or equal to pH 3.5) may be brought back to the required pH using double-strength buffered peptone water, but the pH of such products should be checked when these are tested for the first time to ensure the required range is achieved.

7.2.12 Packaged products

Clean the surface of rigid or semi-rigid packaging using mild detergent in water, then dry with a clean towel or fresh absorbent paper. When packaging is very thin and could be damaged by wetting (eg pieces of food packaged in films or flexible containers), omit this step and disinfect only. Disinfect the outside of packaging carefully with 70 % (volume fraction) alcohol or aseptic wipes to avoid contamination when opening. Open film-wrapped portions of food on trays carefully by peeling off the packaging film so the food can be exposed for sampling. For foods packed in a controlled atmosphere and vacuum-packed foods, open the sealed packaging using a sterile knife, scalpel or scissors and forceps or tongs.
7.2.13 Whole crustaceans such as crabs

Disinfect the surface (using cotton wool with alcohol at a volume fraction of 70 %) and with a sterile hammer, pliers or forceps, remove or break the carapace and claws to extract the maximum amount of flesh for testing. For large claws, an oyster cracker can be used to break open the shell before extracting the flesh.

7.2.14 Inhibitory food materials

For food materials that contain inhibitory substances (eg onion powder, garlic, oregano, peppers, certain teas and coffees, vitamin premixes and highly salted products), it is necessary to decrease the antimicrobial activity before testing by using special preparation procedures such as:

- use of greater dilutions (eg 1 in 100 for cinnamon and oregano and 1 in 1 000 for cloves)
- addition of potassium sulphite (K₂SO₃) to the buffered peptone water to achieve a final concentration of 0.5 % (w/v)
- use of diluent at 37 °C ± 1 °C, to aid dissolution, and higher dilutions (eg 1 in 50) for vitamin premixes
- use of higher dilutions for products containing more than 10 % (mass fraction) salt (sodium chloride) to ensure the total concentration in the initial suspension (not including any salt content of the diluent or enrichment broth) does not exceed 1 % (w/v)

If any of these techniques are used, spiked sample process controls shall be included at first use to verify the effectiveness of the neutralization process chosen.

7.2.15 Cocoa and cocoa-containing products

Use either UHT milk or reconstituted non-fat dry milk powder (100 g/l water; sterilized after reconstitution) as the pre-enrichment broth for detection of the significant pathogens Salmonella spp. and STEC. BPW may be used as a general diluent for other tests.

NOTE: Milk is used to neutralize the bactericidal effect of cocoa or cocoa-containing products. The probable inhibitory factor in cocoa is anthocyanin. Preheat the diluent to 37 °C to 40 °C.

Weigh the test portion (eg 25 g) into a plastic bag, add the warmed diluent (eg 225 ml) to achieve a 1 in 10 initial suspension and mix by hand immediately.
Preparation of samples and dilutions, plating and sub-culture

Leave the suspension at laboratory ambient temperature (18 °C to 27 °C) for 20 - 30 min to melt. Then, mix completely using a stomacher as described above

7.3 Preparation of dilutions

Use peptone saline diluent (PSD) at ambient temperature for all dilutions.

To prepare decimal dilutions (figure 1) transfer 1.0 mL of the initial suspension ($10^{-1}$) to 9.0 mL of PSD avoiding contact between the pipette/pipette tip and the diluent. Mix carefully using a vortex mixer or by hand for 5 – 10 seconds. This constitutes the $10^{-2}$ dilution.

If a 1 in 20 homogenate has been prepared use 2.0 mL of the homogenate and 8.0 mL of diluent to prepare a $10^{-2}$ dilution.

Using a fresh pipette/pipette tip for each dilution, repeat this procedure to produce further decimal dilutions.

Figure 1. Serial dilutions

Information note: The time lapse between preparation of the initial suspension and the beginning of preparation of the further dilutions should not exceed 30 minutes, and the overall time lapse between preparation of the initial suspension and inoculation of the plating media should not exceed 45 minutes.

A fresh sterile tip must be used of each dilution.
7.4 Plating of homogenates

Using a fresh graduated pipette, pipette tip or spiral cup for each dilution inoculate the required volume (eg 50 µL, 0.1 mL, 0.5 mL and/or 1 mL) of the required dilution to the surface of the plating media or to a petri dish for inoculation with tempered media. For official control or formal samples, plate consecutive serial dilutions in duplicate.

Information note: The dilutions, volumes plated and media used are method dependant; please refer to individual methods.

For surface spread plates, use a sterile hockey stick spreader to spread the inoculum over the surface of the plate ensuring that the liquid does not come into contact with the outer edges of the plate as shown in Figure 2.

Information note: If inoculating multiple plates with serial dilutions it is permissible to use the same graduated pipette or pipette tip and hockey stick for inoculation and spreading but only if the operator starts at the highest dilution (most dilute) and works back towards the lowest dilution (most concentrated).

Figure 2. Plating of homogenates

Information note: When inoculating multiple plate types, it is good practice to work in the order of the least selective to the most selective based on media properties.
4.0 Subculture

All sub-culture procedures are performed in a designated area of the laboratory or in a Class I Microbiological Safety Cabinet. The volumes and media used are method dependent please refer to individual methods. Also refer to FNES6 (Q12) Sample Processing and Result Entry in Starlims10.

8.1 Liquid to liquid

Sub-culture from a pre-enrichment stage and enrichment stage is performed for several tests. The procedure to be followed is described below and great care must be taken to ensure that cross contamination of samples does not occur. Wherever possible enrichment cultures from ready to eat samples must be sub-cultured before enrichment cultures from not ready to eat or IQC/ EQA samples, or ideally the work should be carried out in different laboratory areas.

1. For each sample label the secondary enrichment media with the sample number, mix if appropriate (eg MKTTn), loosen the cap and place in a rack.

2. Transfer the enrichment culture (closure bag eg Whirlpak® or honey pot) to the designated area or into the MSC and place in a rack or plastic tray to prevent/contain spillages.

3. Open the closure bag by undoing the wire closures and unrolling the bag or remove the lid from the honey pot.

4. Using a sterile single use pastette or graduated pipette, take up the required volume of culture for sub-culturing (1mL or 100µL=3-4 drops). It is recommended that sub-culturing from a closure bag be performed, using a long bodied disposable pastette.

Information note: If using disposable pastettes the volume dispensed must be determined and the number of drops used must represent a volume as close to 100µL as possible (3-4 drops).

5. Remove the lid of the secondary enrichment media container and transfer the required volume.

6. Replace the lid.

7. Close the primary enrichment container by rolling down the enrichment culture bag, expelling any air, and securing the wire closures or by replacing the lid of the honey pot.

8. Primary enrichment broths are retained until the test is complete and are disposed of in accordance with local waste disposal procedure.

9. Secondary enrichment broths are incubated as described in specific methods.
8.2 Liquid to solid

Sub-culture from a primary or secondary enrichment stage to a plate media is performed for several tests. The procedure to be followed is described below and great care must be taken to ensure that cross contamination of samples does not occur. Wherever possible enrichment cultures from ready to eat samples must be sub-cultured before enrichment cultures from not ready to eat samples, IQC and EQA samples or ideally the work should be performed in different laboratory areas.

For each sample label the appropriate plate media with the sample number, the test stage (eg Primary (1), Secondary (2), 24hrs sub (24), 48hr sub (48), MKTTn (KT), RV (RV)).

1. Transfer the enrichment culture (closure bag or universal container) to the designated area or into the MSC and place in a rack or plastic tray to prevent/contain spillages.

2. Open the closure bag by undoing the wire closures and unrolling the bag or remove the lid from the universal container.

3. Using a sterile single use loop of the appropriate size as described in individual methods (See Figure 3, loop 1) dip this into the enrichment culture and inoculate a small area of the plate (see Figure 3). Discard this loop as for contaminated waste.

4. Using a fresh 10µL loop or sterile stick (Figure 3, Loop 2) spread for single colonies as shown in Figure 3.

5. Roll down the enrichment culture bag expelling any air and secure the wire closures or replace the lid if subbing from a universal.

6. Primary and Secondary enrichment broths are retained until the test is complete and are disposed of in accordance with the local procedure for disposal of laboratory waste.

7. Inoculated plates are incubated as described in specific methods.
8.3 Solid to solid

Any colony that is sub-cultured for the purpose of confirmation must be sub-cultured in such a way as to ensure isolated colonies are produced. This enables identification of mixed cultures. If discrete colonies are not achieved, re-plating of isolates will be required before further confirmation is carried out. Typically, 5 colonies are sub-cultured to a single plate and this should be done using fresh 1 µL loops for the initial inoculation followed by plating for single colonies using fresh 10 µL loops as shown in Figure 4.

Information note: When inoculating multiple plate types, it is good practice to work in the order of the least selective to the most selective based on media properties.

Information Note: For Listeria confirmation it is necessary to stab the agar with the initial inoculum as this enhances development of haemolysis.
Figure 4. Subculture of colonies for confirmation.

A fresh sterile loop must be used for each colony.

5.0 Quality control

Further quality control of media and internal quality assurance checks should be performed according to in-house procedures using the strains specified in individual test procedures.
6.0 Acknowledgements and contacts

This Standard Method has been developed, reviewed and revised by National Infection Service, Food, Water and Environmental Microbiology Methods Working Group.

The contributions of many individuals in Food, Water and Environmental laboratories, reference laboratories and specialist organisations who have provided information and comment during the development of this document are acknowledged.

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References


Table 1: Diluents for use in sample preparation of specific products

<table>
<thead>
<tr>
<th>Product</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>peptone/saline</td>
</tr>
<tr>
<td>Liquid milk products</td>
<td>peptone/saline</td>
</tr>
<tr>
<td>Cheese &amp; processed cheese</td>
<td>sodium citrate or di-potassium hydrogen phosphate pH 7.5</td>
</tr>
<tr>
<td>Frozen milk products including edible ices</td>
<td>peptone/saline (room temperature)</td>
</tr>
<tr>
<td>Butter</td>
<td>peptone/saline (pre-warmed to about 45°C)</td>
</tr>
<tr>
<td>Fermented milk, yoghurt, sour cream</td>
<td>di-potassium hydrogen phosphate pH 7.5</td>
</tr>
<tr>
<td>Dried milk</td>
<td>di-potassium hydrogen phosphate pH 7.5</td>
</tr>
<tr>
<td>Dried sweet whey, dried buttermilk, lactose</td>
<td>peptone/saline</td>
</tr>
<tr>
<td>Acid casein, lactic casein acid whey powder</td>
<td>di-potassium hydrogen phosphate pH 8.4</td>
</tr>
<tr>
<td>Caseinate, rennet casein</td>
<td>di-potassium hydrogen phosphate pH 7.5</td>
</tr>
<tr>
<td>Chocolate and chocolate confectionary</td>
<td>Peptone Saline Diluent (pre-warmed to 40°C)</td>
</tr>
</tbody>
</table>