Enumeration of β-glucuronidase positive Escherichia coli: Pour plate method

Microbiology Services
Food Water and Environmental Microbiology
Standard Method

FNES3 [F8]

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Methods Working Group
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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.

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<table>
<thead>
<tr>
<th>Controlled document reference</th>
<th>FNES3 (F 8)</th>
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<tbody>
<tr>
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</tbody>
</table>

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<thead>
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<th>Section(s) involved</th>
<th>Amendment</th>
</tr>
</thead>
<tbody>
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<td>Table of differences to ISO</td>
<td>Clarification of plating procedure and justification for not carrying out duplicate testing of all samples.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>3.2 Specific safety considerations</td>
<td>Microwave added and text colour changed to highlight hazards and controls</td>
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<td></td>
<td>7</td>
<td>4.0 Equipment</td>
<td>Option for use of microwave included</td>
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<td></td>
<td></td>
<td></td>
<td>7</td>
<td>5.0 Media and reagents</td>
<td>Statement added regarding use of other diluents.</td>
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<tr>
<td></td>
<td></td>
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<td>8</td>
<td>6.2 Sample preparation</td>
<td>Molten tempered agar added within 15 minutes of transferring to the plate.</td>
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<td></td>
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<td>9</td>
<td>7.0 Quality control</td>
<td><em>Klebsiella aerogenes</em> name changed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>8.0 Calculating results</td>
<td>Estimated count comment revised, reference to Starlims added</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>10</td>
<td>9.0 Reporting of results</td>
<td>Reference to Starlims added</td>
</tr>
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<td></td>
<td>13</td>
<td>References</td>
<td>Updated</td>
</tr>
</tbody>
</table>
Enumeration of β-glucuronidase positive *Escherichia coli*: pour plate method

**Introduction**

**Scope**

The method described is applicable to the enumeration of β-glucuronidase-positive *Escherichia coli* in food and dairy products. It must be used if samples are being tested for Official Control purposes by the method laid down in Commission Regulation (EC) No 2073/2005\(^1\) (amended by 1441/2007)\(^2\) on microbiological criteria for foodstuffs. It is applicable for the enumeration of *E. coli* in all foods and dairy products and must be used for testing of minced meat; mechanically separated meat; meat preparations; cheeses made from milk or whey that has undergone heat treatment; butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation (thermised); ready to eat (RTE) pre-cut fruit and vegetables and RTE unpasteurised fruit and vegetable juice. This method can also be used for the enumeration of β-glucuronidase positive *E. coli* in environmental samples such as swabs and cloths taken from the area of food production or food handling. It uses a colony count technique at 44°C with a solid medium containing a chromogenic ingredient for detection of the enzyme β-glucuronidase.

**Information Note:** Strains of *E. coli* which do not grow at 44°C and, in particular, those that are β-glucuronidase negative, such as *E. coli* O157, will not be detected using this method.

**Background**

The presence of *E. coli* in food is regarded as an indication of contamination with organisms of faecal origin. Commission Regulation (EC) No 2073/2005\(^1\) (amended by 1441/2007)\(^2\) on microbiological criteria for foodstuffs lays down different acceptance criteria dependent on the type of food tested.

The method described is based on BS ISO 16649-2:2001\(^3\) and describes the enumeration of β-Glucuronidase positive *E. coli* by pour plate. This method differs from BS ISO 16649-2:2001 in the following ways:

<table>
<thead>
<tr>
<th>PHE method F8</th>
<th>BS ISO 16649-2:2001</th>
<th>Justification for variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scope</td>
<td>Includes all food and environmental samples</td>
<td>Does not include environmental samples</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>1 mL of sample used for duplicate testing of official and formal samples only.</td>
<td>Recommends duplication and further dilution for all samples</td>
</tr>
</tbody>
</table>
# Enumeration of β-glucuronidase positive *Escherichia coli*: pour plate method

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>PHE method F8</th>
<th>BS ISO 16649-2:2001</th>
<th>Justification for variation</th>
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<tbody>
<tr>
<td></td>
<td>Specifies incubation of selective agar plates at 37°C for 4 ± 1 h followed by 44°C for 21± 3 h.</td>
<td>Specifies incubation of selective agar plates at 44°C for 21 ± 3 hours or if the presence of stressed cells is suspected, then incubation at 37°C for 4±1 h followed by 44°C for 21±3 h.</td>
<td>Presence of stressed cells may be suspected in all processed foods. Therefore, for simplicity and consistency, the requirement for the cycling incubation stage has been included for all samples.</td>
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</table>

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.
1.0 Principle

The enumeration of *E. coli* by this colony count technique involves the inoculation of pour plates and mixing of specified volumes of the sample or dilutions of the sample with a cooled molten selective culture medium containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (BCIG). Incubation is at 37 °C for 4 h followed by 44 °C for 21 h to allow for the selective growth of *E. coli*. Detection of *E. coli* colonies uses the chromogenic substrate BCIG to detect β-glucuronidase activity through the generation of blue colonies. The number of colony forming units (CFU) of β-glucuronidase-positive *E. coli* per gram (g) or per millilitre (mL) of sample is calculated. β-glucuronidase activity in Gram negative bacteria is restricted to *E. coli* (90 %), *Shigella* species, *Yersinia* species and *Salmonella* species.

2.0 Definitions

*For the purpose of this method the following definition applies:*

**β-glucuronidase-positive** *Escherichia coli*
Bacteria that grow at 44°C to form a typical blue colony on tryptone bile glucuronide medium (TBX) under the conditions specified in this method.

**Enumeration of β-glucuronidase-positive** *E. coli*
Determination of the number of colony-forming units (CFU) of β-glucuronidase-positive *E. coli*, per millilitre or per gram of sample, when tests are carried out and calculations performed in accordance with this method.

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 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

Care must be taken when using a boiling water bath, steamer or microwave to melt the agar medium used in this procedure. Appropriate safety measures should be in place, and PPE must be made available (eg face visor; water-proof, heat-proof gloves) according to a local risk assessment.

3.3 Laboratory containment

All procedures can be performed in a Containment Level 2 Laboratory.

4.0 Equipment

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1g
- Stomacher
- Gravimetric diluter (optional)
- Vortex mixer
- Automatic cycling incubator: 37 ± 1°C and 44 ± 1°C
- Boiling water bath, steamer or microwave (optional)
- Water-bath, capable of being maintained at 44-47°C
- Colony counter (optional)
- Stomacher bags (sterile)
- 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)
- Petri dishes, of approximately 90cm in diameter

5.0 Culture media and reagents

Equivalent commercial dehydrated media may be used; follow the manufacturer’s instructions.
Enumeration of β-glucuronidase positive *Escherichia coli*: pour plate method

**Peptone saline diluent (Maximum recovery diluent)**

Peptone 1.0 g
Sodium chloride 8.5 g
Water 1L
pH 7.0 ± 0.2 at 25°C

**Buffered peptone water**

Enzymatic digest of casein 10.0 g
Sodium chloride 5.0 g
Disodium hydrogen phosphate dodecahydrate 9.0 g
or anhydrous disodium hydrogen phosphate 3.5 g
Potassium dihydrogen phosphate 1.5 g
Water 1L
pH 7.0 ± 0.2 at 25°C

**Tryptone Bile Glucuronide agar (TBX)**

Enzymatic digest of casein 20.0 g
Bile Salts No.3 1.5 g
5-bromo-4-chloro-3-indoyl-β-D-galacturonic acid (BCIG) cyclohexylammonium salt 75mg
Dimethyl sulfoxide (DMSO) 3 mL
Agar 15.0 g
Water 1L
pH 7.0 ± 0.2 at 25°C

**Information Note**: Additional diluents may be required for dairy products please refer to SOP D1 for media formulations.

6.0 Sample processing

6.1 Sample preparation and dilutions

Following the procedure described in Standard Method F2 – Preparation of Samples and Dilutions prepare a 10⁻¹ homogenate in either peptone saline diluent (PSD) or buffered peptone water and further decimal dilutions as required in PSD. For dairy products, appropriate diluents for the preparation of a 10⁻¹ homogenate are given in Standard Method D1 – Preparation of Samples and Decimal Dilutions. For swabs refer to Standard Method E1- Detection and Enumeration of Bacteria in Swabs and Other Environmental Samples.
Homogenise for between 30 seconds and 3 minutes in a stomacher. The homogenisation time required will depend on the manufacturer instructions and the type of sample being examined.

6.2 Inoculation and incubation

Using a sterile pipette, aseptically transfer to a sterile Petri dish 1mL of undiluted (10^0) test sample (if liquid), or 1mL of the initial dilution (10^{-1}) in the case of other products. If required repeat the procedure with further decimal dilutions, using a new sterile pipette for each dilution. If testing samples for the purpose of official control or in the case of formal samples inoculate all dilutions in duplicate.

Pour into each Petri dish 15-18mL of molten TBX medium that has previously been melted and cooled to 44 - 47°C in a water bath. Immediately after pouring, carefully mix the inoculum with the medium to obtain evenly dispersed colonies after incubation and allow the mixture to set by standing the Petri dishes on a cool horizontal surface. Mixing of the medium and the test portion must be performed within 45 minutes of preparation of the sample homogenate and within 15 minutes of transfer of the sample portion to the Petri dish.

Invert the inoculated dishes so that the bottom is uppermost and place them in an incubator set at 37 ± 1°C  for 4 ± 1 h and then at 44 ± 1°C for 21 ± 3 h. The total incubation time must not be longer than 24 h.

6.3 Counting of colonies

After incubation count the typical colonies of β-glucuronidase positive *E. coli* in each dish containing less than 150 typical colonies and less than 300 total (typical and non-typical) colonies. Typical colonies are blue and will be of various shapes within the three dimensional structure of the agar medium. Non typical colonies will be pale blue.

If non-typical colonies are found in a Petri dish retained for counting, these colonies should be taken into consideration during calculation as defined in section 8.0.

6.4 Confirmatory tests

No confirmatory tests are performed. Bacteria that grow in TBX agar at 44°C with a positive β-glucuronidase reaction (blue colonies) are considered to be *E. coli*.
7.0 Quality control

Quality control of media and internal quality assurance checks should be performed according to in-house procedures using the following control organisms:

Positive control
E. coli NCTC 9001
E. coli NCTC 13216 (Weak β-glucuronidase reaction)

Negative control
Raoultella planticola NCTC 9528

Each batch of tests should include a positive and negative control.

8.0 Calculation of results

Calculations occur automatically in the STARLIMS system as describe in Method Q12 Sample processing and result entry in STARLIMS\textsuperscript{11}. Calculations are performed as described below.

8.1 Calculation of results from routine samples

Calculate the number of β-Glucuronidase Positive E.coli per g as follows:

\[
\text{Count per g} = \frac{\text{Count}}{\text{Volume tested} \times \text{dilution}}
\]

8.2 Calculation of results from formal or official control samples

For a result to be valid, it is considered necessary to count at least one dish containing a minimum of 15 colonies. Use the plate counts to calculate N, the β-Glucuronidase Positive E.coli present in the test sample per millilitre or per g, as the weighted mean from two successive dilutions using the following equation:

\[
N = \frac{\Sigma a}{V (n_1 + 0.1n_2) d}
\]
when:

\[ \sum a \quad \text{is the sum of the colonies counted on all the plates retained from two successive dilutions, at least one of which contains a minimum of 15 CFU} \]

\[ n_1 \quad \text{is the number of plates counted at the first dilution} \]

\[ n_2 \quad \text{is the number of plates counted at the second dilution} \]

\[ d \quad \text{is the dilution from which the first counts were obtained [d = 1 in the case (liquid products) where the directly inoculated test sample is retained, d = 0.1 for } 10^{-1} \text{ dilution etc]} \]

\[ V \quad \text{is the volume of the inoculum, in millilitres, applied to each plate} \]

Round off the result to two significant figures.

### 8.3 Estimation of counts in formal or official control samples (low numbers)

If both dishes at the level of the first retained dilution contain less than 15 confirmed colonies, calculate \( N_E \), the estimated number of β-Glucuronidase Positive \( E.\text{coli} \) present in the test sample, as the arithmetical mean from two parallel plates using the following equation:

\[
N_E = \frac{\sum a}{V \cdot n \cdot d}
\]

when:

\[ \sum a \quad \text{is the sum of the confirmed colonies counted on the two plates} \]

\[ n \quad \text{is the number of plates retained} \]

\[ d \quad \text{is the dilution from which the first counts were obtained [d = 1 in the case (liquid products) where the directly inoculated test sample is retained, d = 0.1 for } 10^{-1} \text{ dilution etc]} \]

\[ V \quad \text{is the volume of the inoculum, in millilitres, applied to each plate} \]

**Information Note:** *Estimated counts (8.3) should be reported with the comment “Count Estimated due to low number”*
Enumeration of β-glucuronidase positive *Escherichia coli*: pour plate method

9.0 Reporting of results

All results are reported using the STARLIMS system as described in method Q13 Technical Validation and release of result in STARLIMS. The test report must specify the method used, all details necessary for complete identification of the sample and details of any incidents that may have influenced the result.

Take as the result the number of β-glucuronidase-positive *E. coli* per mL (liquid products) or per g (other products) expressed as a whole number to two significant figures (if less than 100).

If count is 100 or more, report counts with one figure before and one figure after the decimal point in the form of:

\[ a \times 10^b \text{ CFU per g or mL} \]

where a is never less than 1.0 or greater than 9.9 and b represents the appropriate power of ten. Round counts up if the last figure is 5 or more and down if the last figure is 4 or less.

eg: 1920 CFU per g = 1.9 x 10^3 CFU per g

235,000 CFU per g = 2.4 x 10^5 CFU per g

9.1 Lower detection limit

**Liquid products**

If plates prepared from the undiluted (10^0) product are found to contain no colonies, report the result as

*E.coli* (β-Glucuronidase positive) Not Detected CFU per mL

**Solid food products**

If plates prepared from the 10^-1 dilution of the product contain no colonies report the result as

*E.coli* (β-Glucuronidase positive) Less than 10 CFU per mL or g

**Swabs and cloths**

The lower limit of detection may vary, depending on the quantity of diluent used in the preparation of the sample. Care must be taken when reporting these results to ensure that the appropriate dilution factor (d) is used in the calculation of results. Calculation of results for swabs and other environmental samples is described in method E1^10.
Overgrowth of non-target organisms at lower dilutions may mean that a lower limit of detection of 10 cannot be reported. In this event a “less than” result from the lowest dilution with a countable results must be reported eg less than $1 \times 10^2$ CFU assuming 1.0 mL inoculum of the $10^{-2}$ dilution. In the unlikely event that a lower limit cannot be determined the result must be reported as “VOID due to overgrowth of non-target organisms”, unless re-testing with further dilutions has been carried out within 36 h of sample collection.

9.2 Upper detection limit

If there are only plates containing more than 300 colonies report as greater than the upper limit for the test dilution used with the comment “Count too high to be estimated at dilution used”.

10.0 Reference facilities and referral of cultures

It is not necessary to refer cultures for further investigation.

11.0 Acknowledgements and contacts

This Standard Method has been developed, reviewed and revised by Microbiology Services, 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


Appendix: Flowchart showing the process to perform enumeration of β-glucuronidase positive *Escherichia coli*: pour plate method

1. Prepare a $10^{-1}$ dilution of sample.

2. Homogenise by stomaching.

3. If necessary prepare further dilutions in peptone saline diluent. If sample is formal or an official sample perform testing in duplicate.

4. Subculture 1 mL of each dilution into sterile Petri dishes as required. Pour 15-18mL of molten TBX agar into plates and mix. Allow to set.

5. Incubate at 37°C for 4 hours and 44°C for 18 h.

6. Count typical and non-typical blue colonies.

7. Calculate the result and report.