# FAO Reference Centre for Bivalve Mollusc Sanitation

## Generic protocol - Enumeration of *Escherichia coli* in bivalve molluscan shellfish by the most probable number (MPN) technique (based on ISO 16649-3)

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Cefas

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**History of Procedure**

| Issue | Date | Section | Changes |
|---|---|---|---|
| 1 | 29.06.20 | All | First issue |
| 2 | 26.03.21 | 2 | Paragraph giving information on protocols application |
| 3 | 03.10.23 | 3 | Note regarding MPN calculator and MPN tables |
| | | 5 | Equipment for dissection of gastropods and echinoderms added |
| | | 8.4 | Harmonisation of sample preparation, addition of oyster cracker image and information on how to prepare gastropods and echinoderms |
| | | 8.5 | Procedure for blending and stomaching harmonised with the UK NRL protocol |
| | | Appendix 1 | Species list updated |
| | | References | Updated References |
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# Contents

# 1. Introduction

The consumption of sewage polluted bivalve molluscs may cause illness in the consumer through ingestion of pathogens such as *Salmonella*, norovirus and hepatitis A virus. The risks of exposure to these infectious agents are compounded by the traditional consumption of raw, or only lightly cooked bivalve molluscs. Enteric bacteria, such as *Escherichia coli* (*E. coli*) are used as indicator organisms to assess the sanitary quality of bivalve molluscs and their growing waters and to predict the risk of exposure to enteric pathogenic microorganisms.

Codex Standard 292-2008 (2015 revision; Codex Alimentarius, 2015) identifies the use of the most probable number (MPN) method specified in ISO 16649-3 ('Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide') or equivalent for the enumeration of *E. coli* in live or raw bivalve molluscs. The method is also recommended in the FAO/WHO Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes (FAO/WHO, 2021) and is the reference method for the enumeration of *E. coli* in bivalve molluscs and other marine shellfish species including gastropods and echinoderms under EU food hygiene legislation for both classification (European Union, 2019) and end product testing purposes (European Union, 2015).

# 2. Scope

This protocol has been produced with reference to ISO 16649-3 and selected parts of other relevant ISO standards referenced in Section 11. The theoretical limit of detection is an MPN of 18 *E. coli* per 100 g of flesh. In the context of this protocol, *E. coli* produces acid from lactose at 37±1 ˚C and expresses β-glucuronidase activity at 44±1 ˚C.

Laboratories using this protocol to examine samples in support of a sanitation programme intended to comply with official controls for the application of food and feed law with regard to live bivalve molluscs for the European Union should ensure that the relevant parts of the following references are satisfied.

**Note:** In addition to bivalve molluscs, this method is also applicable to analysis of other marine shellfish species including gastropods and echinoderms. In these cases alternative sample preparation methods should be used – these are detailed in section 8.4.

**Note:** The 5x3 and 5x4 MPN tables included in this document have been generated using the MPN calculator referenced in ISO 7218:2007/Amd1:2013. Values have been converted from MPN/g to MPN/100g. A shellfish-specific MPN calculator has been developed and empty and prepopulated versions are available on the FAO Reference Centre website (Technical Guidance and Calculation Spreadsheets).

# 3. Principle

This protocol for the enumeration of *E. coli* in bivalve molluscs and other shellfish is based on a two-stage, five-tube (or bottle), three-dilution MPN format (although additional dilutions can be used). The first stage of the method is a resuscitation step requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted shellfish homogenates and incubation at 37±1 ˚C

for 24±2 hours. The presence of *E. coli* is confirmed by sub-culturing tubes or bottles showing acid production onto agar containing 5-bromo-4-chloro-3-indolyl-β-D glucuronide and detecting β-glucuronidase activity. Additional dilutions may be required to give a required end-point. The results are expressed as the Most Probable Number (MPN) per 100 g shellfish.

**Note:** Strains of *E*. *coli* that do not grow at 44±1 ˚C and those that are β-glucuronidase negative, such as *E. coli* O157 and some other strains of pathogenic *E. coli,* will not be detected by this protocol.

# 4. Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before using this protocol. Risks of cuts and minor physical injury exist when performing this procedure, particularly when using sharp oyster knifes to open shellfish. Appropriate measures to reduce these risks should be taken. Homogenisation of shellfish should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. Laboratory procedures should conform to the recommendations given in the WHO Laboratory Biosafety Manual Third edition (WHO, 2004) or relevant national legislation or guidelines.

# 5. Equipment

- Autoclave (or media preparator including an autoclave component)
- Drying cabinet or ventilated oven capable of being maintained between 25±1 °C and 50±1 °C
- Balance capable of weighing to ±0.1 g and reference weights for calibration
- pH-meter, with automatic or manual temperature compensation and having a resolution of 0.01 pH units and accurate to within ±0.1 pH unit at 25 °C. Reference solutions for calibrating the pH meter
- Local Exhaust Ventilation (LEV; for weighing powders during media preparation)
- Class II safety cabinet
- Thermometer with a resolution of 0.1 °C or better at the temperature being measured
- Sterile measuring cylinder – 250 ml (a larger cylinder may be needed for some species)
- Sterile glass tubes or screw-topped bottles capable of holding 20 ml volumes (for liquid media)
- Sterile petri dishes (for solid media): diameter 90 mm; height 15-16 mm; vented
- Shucking knife, oyster cracker or other suitable equipment for opening bivalve molluscs
- Dissecting equipment for gastropods and echinoderms – Sterile tray, forceps, scissors, spatula and hammer or equivalent tools
- Food grade plastic bags
- Absorbent paper towel
- Weighing dish capable of holding the largest size of bivalve mollusc to be tested plus released intravalvular fluid. Must be capable of being cleaned and sterilized (for use with oyster cracker)
- Safety gloves – for example a chain mail glove (for use with the shucking knife)
- Waring blender and 1 litre blending jars or stomacher and stomacher bags
- Refrigerators at 3±2 ˚C and 5±3 ˚C
- Sterile container of at least 500 ml capacity (A larger capacity may be required when large bivalve molluscs are to be examined. Examples include *Panopea generosa* (geoducks), *Mya arenaria* (soft shell clams; sand gapers) and some types of razor clams)

- Safety Bunsen burner or electric micro-incinerator
- Protective gloves – single use
- Incubator or recirculating water bath at 37±1 ˚C and 44±1 ˚C [1]
- Platinum or sterile disposable loops - 10 µl (or 1 µl; see Section 8.7)
- Pipette filler and graduated pipettes or automatic pipettor and pipette tips of a range of sizes e.g. 1ml and 10ml. For handling mixed shellfish homogenates (1:3 and $10^{-1}$ dilution) open-ended pipettes may be necessary due to high concentrations of particulate matter.

# 6. Media [2]

Media stored under refrigeration should be allowed to equilibrate at room temperature before use (ISO 11133). If necessary, dry the surface of plated media before use. For performance testing of media see Table 1 in section 7.

**Note:** ISO 16649-3 contains further details for the preparation of MMGB and TBGA from the basic ingredients. Dehydrated media purchased commercially should be prepared according to the manufacturer's instructions.

- Ethanol
- 0.1 % peptone (0.1 % P); formula per litre - de-ionised water 1±0.01 litre, peptone bacteriological 1±0.1 g. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5±3 °C in the dark.
- Peptone salt solution (PSS); formula per litre - de-ionised water 1±0.01 litre, peptone 1±0.1 g, sodium chloride 8.5±0.1 g, pH 7±0.2. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5±3 °C in the dark.
- Single strength Minerals Modified Glutamate Broth (MMGB); - formula per litre - de-ionised water 1±0.01 litre, ammonium chloride 2.5±0.1 g, sodium glutamate 6.4±0.1 g, minerals modified medium base 11.4±0.1 g, pH 6.7±0.1. Dispense aseptically in 10±0.2 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize by autoclaving at 116 °C for 10 minutes. If not used immediately after cooling to room temperature, store at 5±3 °C in the dark.
- Double strength MMGB; - formula per litre - de-ionised water 1±0.01 litre, ammonium chloride 5±0.1 g, sodium glutamate 12.7±0.1 g, minerals modified medium base 22.7±0.1 g, pH 6.7±0.1. Dispense aseptically in 10±0.2 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize the medium by autoclaving at 116 °C for 10 minutes. If not used immediately after cooling to room temperature, Store at 5±3 °C in the dark.
- Tryptone bile glucuronide agar (TBGA; also marketed commercially as TBX); formula per litre - de-ionised water 1±0.01 litre, tryptone bile x-glucuronide medium 36.6±0.5 g, pH 7.2±0.2. Sterilize by autoclaving at 121 °C for 15 minutes. Cool to 50 °C and pour 18-20 ml of molten medium into sterile Petri dishes and allow to solidify. Store at 5±3 °C in the dark and protected from desiccation for up to four weeks unless results of the laboratory shelf-life validation indicate a longer shelf-life. Plates should be dried before use to remove excess surface moisture (see

[1] In countries where the ambient temperature exceeds the target incubation temperature, an incubator with both heating and cooling units may be required to maintain the required temperature range.

[2] Formulations provided in this generic protocol are based upon the use of Oxoid Ltd listed products for illustrative purposes, alternative suppliers of media and reagents can be used according to the manufacturers' instructions. Laboratories should determine that alternative products are fit for purpose.

ISO 11133 for further considerations in relation to drying). Drying is especially important where TBGA plates are to be subdivided for the inoculation of more than one tube from a single dilution.

# 7. Microbiological reference cultures for performance testing and controls

The FAORC recommends the use of the positive and negative controls throughout the procedure. The strains and criteria included in Table 1 are recommended for use as a minimum.

**Table 1: Microbiological reference strains used for control purposes**

| Media type | Function | Control strain | WDCM [a] | Characteristic reaction | Strain choice [c] |
|---|---|---|---|---|---|
| Mineral-modified glutamate broth (MMGB) | Productivity | *Escherichia coli* | 00012 or 00013 | Acid production. Colour change to yellow | 1 |
| | Selectivity | *Enterococcus faecalis* | 00009 or 00087 | No growth. No colour change | 1 |
| Tryptone bile glucuronide agar (TBGA/TBX) | Productivity (and characteristic reaction) | *Escherichia coli* | 00202 | Blue to blue-green colonies. B-glucuronidase positive (weak) | 2 |
| | | *Escherichia coli* | 00012 or 00013 | Blue to blue-green colonies. B-glucuronidase positive | 1 |
| | Selectivity | *Enterococcus faecalis* | 00009 or 00087 | Total inhibition. No growth | 1 |
| | Specificity | *Pseudomonas aeruginosa* [b] | 00025 | White to beige colonies. B-glucuronidase negative | 1 |
| | | *Citrobacter freundii* [b] | 00006 | | |

[a] Follow the link http://refs.wdcm.org/species.htm to obtain culture collection numbers for WDCM reference strains.
[b] The use of *P. aeruginosa* (WDCM 00025) or *C. freundii* (00006) is given in ISO 16649-3. Following the sub-culturing of *C. freundii* onto TBGA/TBX plates, the former EURL noted growth was not always present. The FAORC recommends the use of *P. aeruginosa* over *C. freundii.*
[c] Reference strains given in ISO 16649-3 for use in performance testing. Strain choice: 1 - Laboratory must select 1 strain as a minimum from the list provided for each media type; 2 - Strain to be used as a minimum.

# 8. Procedures

## 8.1. Sample transport and receipt

Samples must be placed in an intact food-grade plastic bag, or equivalent, and properly packed in a cool box with ice packs – packed in this manner they should reach a temperature between 0 ˚C and 10 ˚C within 4 hours and then maintain this for at least 24 hours [3]. The specific cool box, ice

[3] A temperature data logger may be used to monitor the sample temperature during transit. The data logger must be held in a central position within the cool box and not allowed to come into contact with the ice packs. If the logger has not been

pack and transport condition combination should be validated to ensure that this can be achieved (https://www.cefas.co.uk/nrl/information-centre/nrl-laboratory-protocols/). For samples where less than 4 hours have elapsed between collection from the growing area and receipt at the laboratory, internal air temperature (or between-shellfish sample temperature) should be less than the temperature recorded at the time of sampling. Samples from harvesting areas should be rinsed, but not immersed, and drained at time of sampling. Samples should be regarded as unsatisfactory if on receipt at the laboratory the sample is frozen, the container is leaking, the shellfish are covered in mud or immersed in water or mud/sand.

The sample transport criteria given here are extracted from ISO 6887-3. The use of alternate sample transport criteria may be acceptable, where verification studies have been undertaken and the results of those studies demonstrate that there is no significant effect on the quality of the test results. For samples being taken in support of a growing area sanitation programme, it is recommended that verification studies supporting the use of sample transport criteria outside of the ranges given in ISO 6887-3 are approved by the responsible authority. (See Section 4.3.6 and Annex 13 of the FAO/WHO technical guidance (https://www.fao.org/3/cb5072en/cb5072en.pdf).

**Note:** The UK National Reference Laboratory for foodborne viruses and bacteriological contamination of bivalve molluscs has carried out studies on the effect of extended storage time and elevated temperatures on certain species of bivalve molluscs. The report of this work can be accessed using the following web link https://www.cefas.co.uk/nrl/information-centre/nrl-reports/

## 8.2. Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. Samples should be examined immediately - if storage in the laboratory is necessary, then samples should be stored at 3±2 ˚C and should be processed within 24 hours of collection. If, due to logistical problems, microbiological analysis of samples taken in support of a growing area programme cannot be started within 24 hours of sample collection, a verification study should be undertaken to show that extended storage does not affect the microbiological content of the sample.

## 8.3. Sample selection

Choose shellfish that are alive according to the following criteria:

- Reaction or movement of exposed flesh after touching using a sterile shucking knife
- Shellfish open and close of their own accord
- A tap on the shell causes closing or movement
- Tightly closed shellfish

Discard all dead shellfish and those with obvious signs of damage. Select the appropriate number depending on the species (Appendix 1). More shellfish can be used, if necessary, to produce the required volumes for each analysis.

activated or is found to be in contact with the ice packs on arrival at the laboratory, then the temperature of the shellfish sample itself should be measured using an appropriately calibrated temperature probe.

## 8.4. Sample preparation

Any mud and sediment adhering to the shellfish should be removed prior to opening the shellfish by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish as described below [4].

### 8.4.1. Preparation of bivalve molluscs using the oyster cracker

Sterilise the blade of the oyster cracker before use (see Figure 1 and Figure 2). Place a single animal in a weighing dish on the platform underneath the blade. Lower the lever so that the blade engages the hinge of the shellfish, then fully pull the lever down so that the blade separates the shells. Using a sterilised shucking knife cut the muscle and scrape the meat of both shells into the sterilised container. Transfer any liquor collected in the weighing boat into the sterilised container.

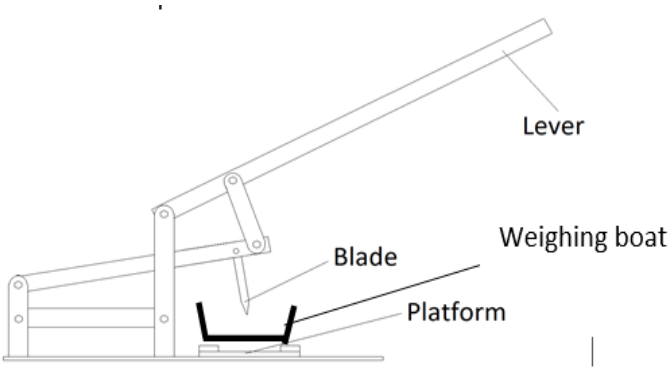



Figure 1: Oyster cracker apparatus

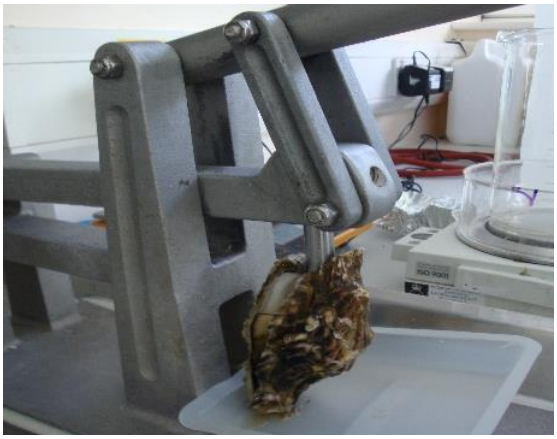


Figure 2: Opening an oyster using an oyster cracker

### 8.4.2. Preparation of bivalve molluscs using a shucking knife

Open all selected shellfish as described below using a sterilised shucking knife or equivalent and empty meat and liquor into a sterile container. If sterilised by heating allow the knife to cool before using. When opening shellfish, ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

#### 8.4.2.1. Oysters and clams

Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain into the sterilised container. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into the sterilised container.

#### 8.4.2.2. Mussels and cockles

Insert the knife in between the shells of the animal and separate the shells with a twisting

[4] Alternative suitable equipment can be used to open bivalve molluscs.

motion of the knife. Collect the liquor from the animal in the sterilised container then cut the muscle between the shells and scrape the contents into the sterilised container.

### 8.4.3. Preparation of gastropods

Place a single animal into the hinge section of the oyster cracker (see Figure 3) and pull the lever down to break open the shell. Transfer the material onto sterile absorbent paper towel on a tray and remove any shell debris using sterile forceps. Repeat the process with the required number of animals (see Appendix 1). Dice the animal flesh using sterile scissors before transferring to a sterile container.

Alternatively, the animals can be opened using a sterile hammer or equivalent tool, without damaging the flesh, by placing the animals in a food grade bag and covering with absorbent paper towel.

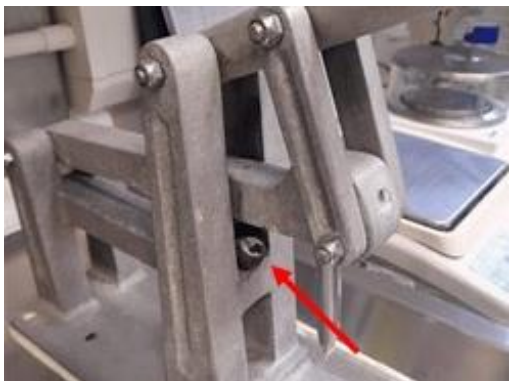


Figure 3: Opening a gastropod using an oyster cracker

### 8.4.4. Preparation of enchinoderms (e.g. sea urchins)

Hold the echinoderm with the mouth parts facing upwards. Using sterile forceps and wearing a strong clean glove, cut out the mouth section (Figure 4) with sterile scissors to expose the flesh inside. Transfer the flesh and internal fluid into a sterile container using a sterile spatula. Repeat the process with the required number of animals (see Appendix 1).

Figure 4: Opening an echinoderm using sterile scissors

## 8.5. Dilution and homogenisation

Weigh the sterilised container and calculate the weight of the contents by subtracting the weight of the pre-weighed container to the nearest gram.

**Note:** ISO 6887-3 allows the use of PSS for the preparation of the primary dilution. FAORC advice is to use 0.1 % P instead at this stage to avoid the addition of extra NaCl.

**Note:** Complete either sections 8.5.1 or 8.5.2.

**The FAORC recommends using a blender and not a stomacher when preparing clam and cockle samples for *E. coli* enumeration. Results from proficiency testing distributions have shown that lower and/or more variable results may be obtained with some clam and cockle species after homogenisation using a stomacher.**

### 8.5.1. Homogenisation in a blender

Measure 2ml of sterile 0.1% P per 1g of shellfish (±2 ml) using a measuring cylinder. Place the contents of the sterilised container into a 1 litre blender jar [5] with the 0.1% P from the measuring cylinder and homogenise at high speed for approximately 1 minute (4 bursts of 15 seconds with at least 5 seconds between bursts) in a Class II safety cabinet. Decant the contents (mixed shellfish homogenate (1:3 dilution)) back into the sterilised container.

Add 30±0.5ml of mixed shellfish homogenate to 70±1ml of 0.1% P to make a master $10^{-1}$ dilution, ensure that this master dilution is thoroughly mixed.

**Note**: it may be necessary to use an open-ended pipette to transfer mixed shellfish homogenate due to high concentrations of particulate matter.

### 8.5.2. Homogenisation using a stomacher

Place the contents of the sterilised container into at least three stomacher bags (e.g. place three stomacher bags inside each other to avoid small pieces of shell from puncturing the bags). Remove excess air from the bags and operate the stomacher for 2-3 minutes. Transfer 50 g of homogenised shellfish into another set of 3 stomacher bags, add 100 ml of 0.1% P and homogenise for a further 2-3 minutes. After homogenisation decant the contents into a new sterilised container and add 350 ml 0.1% P to make the master $10^{-1}$ dilution, ensure that this master dilution is thoroughly mixed.

### 8.5.3. Preparation of further dilutions

Make a further decimal dilution ($10^{-2}$) using the master $10^{-1}$ dilution as prepared in 8.5.1 or 8.5.2 by adding 1ml of the $10^{-1}$ dilution to 9 ml of 0.1% P or PSS. If required, prepare a $10^{-3}$ dilution by adding 1ml of the $10^{-2}$ dilution to 9ml of 0.1% P or PSS.

**Note:** Inclusion of the $10^{-3}$ dilution is dependent on the purpose of testing. It is required where it is necessary to determine values of >18000 MPN per 100g, for example, under the EU classification system the upper limit for Class C samples is 46000 MPN per 100g. Dilutions beyond $10^{-3}$ will only be required in special circumstances (e.g. pollution incident investigations).

**Note:** For samples where more than 3 dilutions are tested, ensure all results from each dilution tested is used to calculate the MPN value in section 8.8.

## 8.6. Inoculation and incubation of primary broth

**Note:** MMGB tubes or bottles should be inoculated within 45 minutes of preparing the initial sample suspension (ISO 7218 Section 9.2.1).

Inoculate five tubes (or bottles) containing 10±0.2 ml of double strength MMGB with 10±0.2 ml of the $10^{-1}$ master dilution (equivalent to 1 g of tissue per tube and referred to as the 'Neat' concentration). Inoculate five tubes (or bottles) containing 10±0.2 ml of single strength MMGB with 1±0.1 ml of the $10^{-1}$ master dilution (equivalent to 0.1g of tissue per tube and referred to as '$10^{-1}$' concentration). Inoculate five tubes (or bottles) containing 10±0.2 ml of single strength MMGB with

[5] If shellfish are particularly small, it may be necessary to use a smaller blender to achieve a consistent homogenate.

1±0.1 ml of the $10^{-2}$ diluted homogenate (equivalent to 0.01g of tissue per tube and referred to as '$10^{-2}$' concentration) and subsequently repeat with any further dilutions.

Prepare a set of controls for each batch of MMGB inoculated; using a 10 µl sterile loop [6], inoculate separate double and single strength MMGB tubes (or bottles) with the control strains (productivity and specificity) listed in Table 1. Inoculate a separate tube (or bottle) of double with an equal volume of diluent (0.1% P or PSS) and leave one tube (or bottle) uninoculated.

Incubate all test and control tubes (or bottles) of MMGB at 37±1 ˚C for 24±2 hours.

## 8.7. Confirmation of *E. coli*

After incubation examine the MMGB tubes (or bottles) for the presence of acid. Acid production is denoted by the presence of any yellow coloration throughout the medium or change from the negative control (see Figure 5). Confirm the presence of *E. coli* in each tube (or bottle) showing acid production by sub-culturing onto the surface of a TBGA plate (whole plate) within 4 hours, using a 10 µl sterile loop, streaking with the objective to obtain single colonies. Also subculture all MMGB control tubes (or bottles) onto separate TBGA plates for each set of tests undertaken. Incubate all TBGA plates at 44±1 ˚C for 21±3 hours in stacks no more than six plates high and separated from each other and the incubator walls by at least 25 mm.

Figure 2. MMGB after incubation showing colour change

**Note:** TBGA plates can be subdivided into 5 segments, with each segment being inoculated from a single presumptive positive tube (or bottle). It is recommended to use separate plates for each dilution level even if there are less than 5 tubes (or bottles) to subculture. When using subdivided plates, it is recommended to use a 1 µl loop for subculture to help avoid overgrowth of target colonies. Comparative work carried out by the FAORC showed that sub-culturing MMGB tubes using a 1µl loop onto TBGA plates divided into 5 segments did not affect the MPN value.

Following incubation, examine the TBGA for the presence of blue or blue-green colonies. Record the results as positive for any shade of blue or blue-green colonies and negative for colonies of any other colour and no growth. See Figure 6, image A for the results of positive and negative reference controls sub-cultured onto individual TBGA plates and Image B using a subdivided plate for separate tubes from a single dilution.

[6] A 1µl loop can be used instead of a 10µl loop as given in ISO 16649-3. The FAORC has shown the change in loop size has no practical consequences on *E. coli* recoveries.

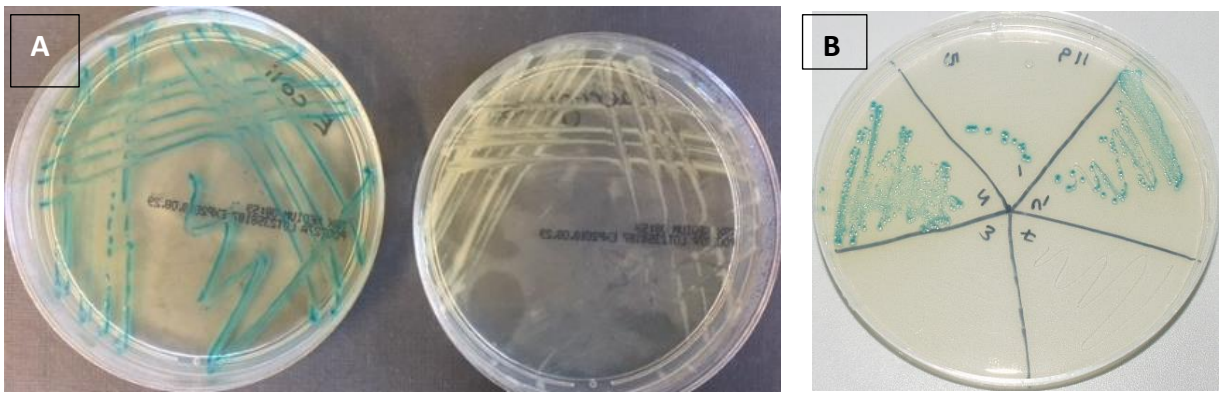



Figure 6. Image A: Positive (blue-green colonies) and negative (cream colonies) subcultures on TBGA. Image B: Results of positive and negative MMGM tubes sub-cultured on a subdivided TBGA plate.

# 8.8. Calculation of *E. coli* Most Probable Number

To determine the MPN, record as negative all MMGB tubes (or bottles) that either do not produce acid after incubation or are negative (i.e. no blue or blue-green colonies) following subculture and incubation on TBGA plates. Record as positive MMGB tubes (or bottles) that produce acid after incubation and are positive (i.e. presence of blue or blue-green colonies) following subculture and incubation on TBGA plates. The total number of tubes (or bottles) at each The number of positive and total tubes (or bottles) at each dilution is used to calculate the MPN value, either by using the Shellfish MPN calculator [7] (see Section 8.8.1) or by referring to the MPN tables given in Appendix 2 (see Section 8.8.2).

**Note:** MPN tube (or bottle) combinations fall into one of three probability categories. 95% of observed tube combinations fall into category 1 with 4% and 1% in categories 2 and 3 respectively. The Shellfish MPN calculator displays the probability category in column 14. Only values determined from combinations that are shown as category 1 or 2 should be recorded. Results for tube combinations that are shown as category 3 values should be recorded as 'Void'. The MPN tables in Appendix 2 only give results for category 1 and 2 combinations; any other combination will be category 3 and results for these tube combinations should be recorded as 'Void'.

## 8.8.1. The Shellfish MPN calculator

Three copies of the Shellfish MPN calculator are available from the FAO Reference Centre website (Technical Guidance and Calculation Spreadsheets). Two have been prepopulated in the general data section with 3 and 4 dilution factors respectively, and a number of 5 MMGB tubes inoculated at each dilution added in the input data section. The third version, suitable for use with e.g. more than 4 dilutions, has no information added.

To use the Shellfish MPN calculators attached to this protocol;

A. Download and open a copy of the appropriate MPN calculator (depending on the number of dilutions tested) from the website.

**Note:** MPN values must be determined using the results obtained from all dilutions tested

[7] The Shellfish MPN calculator has been developed specifically for use in bivalve mollusc official control laboratories. The Excel spreadsheet MPN calculator cited in ISO 7218 (http://standards.iso.org/iso/7218/) can be used to calculate the MPN value. The MPN value calculated using the MPN calculator cited in ISO 7218 is reported as MPN/g and must be converted to MPN/100g (see Appendix 3).

even if lower dilutions are completely negative

B. Click on the ‘Enable content’ button at the top of the spreadsheet.

C. If required, enter relevant data in the general data section. The no. of samples (column 3) and max. no. of dilutions cells (column 4) must be populated if using the generic version of the calculator (these cells are prepopulated in the 3 and 4 dilution factor versions). Name of experiment and date of experiment cells (columns 1 and 2) do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files.

D. If required, enter the total number of tubes at each dilution in the input data section (column 3; these cells are prepopulated in the 3 and 4 dilution factor versions). Sample designation and target organism cells do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files.

E. For each sample, enter the number of positive tubes at each dilution in the input data section (column 4).

F. Press the ‘calculate results’ button in the top right of the spreadsheet or press ‘Ctrl + M’. The calculated MPN value is shown in column 8 as MPN per 100g, and the probability category is shown in column 14.

**Note:** if any data in the input data section is changed, the MPN value will need to be recalculated by re-pressing the ‘calculate results’ button or ‘Ctrl + M’.

### 8.8.2. MPN tables (Appendix 2)

All 3 and 4 dilution MPN tube combinations in probability categories 1 and 2 have been provided in Tables 1 and 2 in Appendix 2. To find the MPN value, refer to the appropriate MPN table for the number of dilutions examined:-

- For 3 dilutions (neat, $10^{-1}$ and $10^{-2}$) use Table 1
- For 4 dilutions (neat, $10^{-1}$, $10^{-2}$ and $10^{-3}$) use Table 2

Find the line in the relevant table that contains the number of positive tubes (or bottles) for each dilution and thus read off the MPN value for that combination of results. The tables only contain category 1 and 2 combinations. Combinations of results that do not appear in the tables are category 3 (see Section 8.9).

**Note:** The entries in the MPN tables given in Appendix 2 are consistent with those obtained with the Shellfish MPN calculator.

## 8.9. Reporting results

Results should be reported as the MPN per 100 g of flesh and intra-valvular liquid. Tube combinations of 0, 0, 0 and 5, 5, 5 should be assigned estimated values of <18 and >18000 MPN per 100g of flesh and intra-valvular liquid respectively (see note). Likewise, tube combinations of 0, 0, 0, 0 and 5, 5, 5, 5 should be assigned estimated values of <18 and >180000. Only values determined from combinations that give a category 1 or 2 profile should be reported. Results for samples with tube combinations that give a category 3 profile should be reported as ‘Void’. Negative samples should be reported as <18 MPN per 100g.

**Note:** According to ISO 7218:2007 + Amd 1:2013 (both the Excel spreadsheet MPN calculator and MPN tables in Annex C (normative)), tube combinations of 0, 0, 0 (or 0, 0, 0, 0) and 5, 5, 5 (or 5, 5, 5, 5) should be given estimated MPN values of 0 and infinity respectively. In bivalve mollusc sanitation programmes it is however common practice for samples yielding all negative tubes (or bottles) to be reported as containing *E. coli* at a concentration less than the nominal lower limit of detection for the range of dilutions used. Likewise, samples yielding all positive tubes (or bottles) have been reported as containing *E. coli* at a concentration above the upper nominal limit of detection for the range of dilutions used. This approach provides additional information of value in assessing data within a bivalve mollusc sanitation programme. The Shellfish MPN calculator and the MPN tables given in Appendix 2 of this protocol follow this practice.

# 9. Uncertainty of test results

Uncertainty inherent in any test method, i.e. instruments, media, analyst performance, etc., can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, through in-house comparability testing between analysts and through external inter-comparability exercises, which would highlight any uncertainties within the test methods.

The inclusion of the measurement of uncertainty (MU) or confidence limits (CL) for the result is not required but it is recommended that laboratories determine, as part of their quality procedure, the MU for the reported results and provide this information on request.

# 10. Quality control

Quality control systems offer a laboratory a mechanism to control preventable inaccuracies within a procedure.

## 10.1. Proficiency testing

It is recommended that laboratories undertaking microbiological examination of samples as part of a bivalve mollusc sanitation programme take part in comparative testing (also known as proficiency testing) relevant to the sample type(s) and method(s). Participation may be required under the sanitation programme (by national legislation or official procedures) or specified by importing countries. Comparative testing provides an indication of performance relative to other participating laboratories and can help improve the performance of the laboratory. Examples of comparative testing for bivalve molluscs are the FAO Reference Centre proficiency testing scheme (see https://www.cefas.co.uk/icoe/seafood-safety/services/proficiency-testing-and-quality-assurance/) and the UK HSA/Cefas Shellfish EQA scheme (for further information contact foodeqa@ukhsa.gov.uk).

## 10.2. Internal Quality controls

Sterility controls should be set up for each container of diluent (0.1% P or PSS) that is used, at the beginning and end of each set of samples that is examined.

Positive and negative controls should be included with each set of examinations (that is, those processed at approximately the same time) and for each medium type (double strength MMGB, single strength MMGB, TBGA). The control strains listed in Table 1 (Section 7) may be used for this purpose. Controls should not be inoculated onto the same TBGA plates as subcultures of MMGB tubes from samples. An uninoculated tube or bottle of double strength MMGB, single strength MMGB and/or plate of TBGA should also be incubated with each set as a sterility control. Where more than one batch of medium has been used for the sample examinations, positive, negative and sterility controls should be included for each batch.

## 10.3. Internal Quality Assurance (IQA)

It is recommended that regular (e.g. monthly) monitoring using known levels of target organism are examined to ensure that routine *E. coli* procedures continue to be effective. Certified reference materials containing a target number of microorganisms (for example Lenticule™ discs) are available from commercial sources for use in internal quality assurance procedures. The target concentration of *E. coli* in samples prepared using reference materials, should be appropriate to the usual range of *E. coli* concentrations expected in routine samples.

# 11. References

Regulations, guidance documents and international standards may be superseded, revised or updated over time. The user of this protocol should make sure that they refer to the most recent version.

Codex Alimentarius, 2015. Standard for Live and Raw Bivalve Molluscs: Codex Stan 292-2008 (Adopted in 2008. Amendment: 2013. Revision: 2014 and 2015). Food and Agriculture Organisation of the United Nations/World Health Organization.

European Union, 2015. Regulation (EU) 2015/2285 of 8 December 2015 amending Annex II to Regulation (EC) No 854/2004 of the European Parliament and of the Council laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption as regards certain requirements for live bivalve molluscs, echinoderms, tunicates and marine gastropods and Annex I to Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L 323, 9.12.2015: 2-4.

European Union, 2019. Commission Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls (Text with EEA relevance.) Off. J. Eur. Union L 131, 17.5.2019: 51–100.

FAO/WHO, 2021. Technical guidance for the development of the growing area aspects of bivalve mollusc sanitation programmes, second edition. Food and Agriculture Organisation of the United Nations/World Health Organization.

ISO 11133. Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media. International Organization for Standardization: Geneva, Switzerland.

ISO 7218. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1. International Organization for Standardization: Geneva, Switzerland.

ISO 16649-3. Microbiology of food chain - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. International Organization for Standardization: Geneva, Switzerland.

ISO 6887-1. Microbiology of food chain – 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. International Organization for Standardization: Geneva, Switzerland.

ISO 6887-3. Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 3: Specific rules for the preparation of fish and fishery products. International Organization for Standardization: Geneva, Switzerland.

WHO, 2004. Laboratory biosafety manual – third edition. Geneva: World Health Organization. 178pp. http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

# 12. Appendices

## 12.1. Appendix 1: Sample sizes of shellfish required for *E. coli* analysis

The following sub-sample sizes are recommended for inclusion in the homogenisation step (the recommended number for sampling is 10 % greater to allow for morbidity in a proportion of animals on receipt at the laboratory):

| Type | Common name [8] | Scientific name [9] | Sample size [10] |
|---|---|---|---|
| Scallops | Mediterranean scallop | *Pecten jacobaeus* | 10 - 12 |
| | King (Great Atlantic) scallop | *Pecten maximus* | 10 - 12 |
| | Queen scallop | *Aequipecten (Chlamys) opercularis* | 15 - 30 |
| | Variegated scallop | *Mimachlamys (Chlamys) varia* | 10 - 18 |
| Oysters | Pacific oyster | *Magallana (Crassostrea) gigas* | 10 - 18 |
| | Portuguese oyster | *Magallana (Crassostrea) angulata* | 10 - 18 |
| | European flat oyster | *Ostrea edulis* | 10 - 18 |
| Mussels | Blue or common mussel | *Mytilus edulis* | 15 - 30 |
| | Mediterranean mussel | *Mytilus galloprovincialis* | 10 - 30 |
| | Northern horse mussel | *Modiolus modiolus* | 10 - 12 |
| | Bearded horse mussel | *Modiolus barbatus* | 15 - 30 |
| Ark Clams | Ark clam | *Barbatia barbata* | 15 - 25 |
| | Noah's ark shell | *Arca noae* | 15 - 30 |
| Clams | Smooth clam | *Callista chione* | 10 - 30 |
| | Striped venus clam | *Chamelea gallina* | 40 - 80 |
| | Rayed artemis | *Dosinia exoleta* | 18 - 35 |
| | Hard clam | *Mercenaria mercenaria* | 12 - 18 |
| | Palourde (Grooved carpet shell) | *Ruditapes (Tapes/Venerupis) decussatus* | 18 - 35 |
| | Manila clam | *Ruditapes (Tapes) philippinarum* | 18 - 35 |
| | Banded carpet shell | *Polititapes (Venerupis) rhomboides* | 20 - 25 |
| | Pullet carpet shell | *Venerupis corrugata (senegalensis)* | 20 - 25 |
| | Warty venus clam | *Venus verrucosa* | 15 - 30 |
| | Atlantic surf clam (Thick trough shell) | *Spisula solida* | 30 - 50 |
| | Cut trough shell | *Spisula subtruncata* | 70 - 90 |
| | Bean clam | *Donax spp.* | 30 - 50 |
| | Wedge shell clam | *Donax trunculus* | 40 - 80 |
| | Common cockle | *Cerastoderma edule* | 30 - 50 |
| | Soft shell clam (Sand gaper) | *Mya arenaria* | 10 - 12 |

[8] Species of relevance provided by European NRLs.
[9] Scientific names are those used by the World Register of Marine Species at the time of writing, however commonly used alternatives for genus or species names are shown in brackets.
[10] Number of shellfish to be tested for *E. coli.* ISO 6887-3 specifies a minimum of 10 animals of any species should be examined. Sample sizes given in this table have been provided by European NRLs. The weight of shellfish flesh and liquor should be at least 50g for the *E. coli* method (for very small species such as *Donax* spp. a minimum amount of 25g is permitted). For species not given in the table, sufficient shellfish should be opened to achieve this minimum weight of flesh and liquor, with the provision that a minimum of ten animals should be used for very large species. In general, the more shellfish that are included in the initial homogenate, the less the final result will be influenced by the inherent animal-to-animal variation in *E. coli* concentration.

| | | | |
|---|---|---|---|
| Razor Clams and Geoducks | Pacific geoduck | *Panopea generosa* | 10 - 12 |
| | Sword razor | *Ensis ensis* | 10 - 12 |
| | Razor shell | *Ensis magnus (arcuatus)* | 10 - 12 |
| | Atlantic razor clam (Jacknife clam) | *Ensis leei* | 10 - 12 |
| | Pod razor | *Ensis siliqua* | 10 - 12 |
| | Grooved razor shell | *Solen marginatus* | 10 - 12 |
| Gastropods | Abalone | *Haliotis spp.* | 10 - 12 |
| | Turbinate monodont | *Phorcus turbinatus* | 30 - 50 |
| | Whelk | *Buccinum undatum* | 10 - 15 |
| | Dog winkle | *Stramonita (Thais) haemastoma* | 15 -25 |
| | Common periwinkle | *Littorina littorea* | 30 - 50 |
| Echinoderms | Purple sea urchin | *Paracentrotus lividus* | 10 - 30 (adults), 40 - 80 (juveniles) |

**Note:** Laboratories are encouraged to submit suggestions on minimum sample sizes for other species to the FAORC. Please note that these recommendations must conform to the minimum requirement of 10 animals given in ISO 6887-3.

# 12.2. Appendix 2: *E. coli* MPN tables

## 12.2.1. Table 1: MPN table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g.

| 1g | 0.1g | 0.01g | MPN/100g | Category |
|---|---|---|---|---|
| 0 | 0 | 0 | <18 [11] | 1 |
| 0 | 1 | 0 | 18 | 1 |
| 1 | 0 | 0 | 20 | 1 |
| 1 | 0 | 1 | 40 | 2 |
| 1 | 1 | 0 | 40 | 1 |
| 1 | 2 | 0 | 61 | 2 |
| 2 | 0 | 0 | 45 | 1 |
| 2 | 0 | 1 | 68 | 2 |
| 2 | 1 | 0 | 68 | 1 |
| 2 | 1 | 1 | 92 | 2 |
| 2 | 2 | 0 | 93 | 1 |
| 3 | 0 | 0 | 78 | 1 |
| 3 | 0 | 1 | 110 | 1 |
| 3 | 1 | 0 | 110 | 1 |
| 3 | 1 | 1 | 140 | 2 |
| 3 | 2 | 0 | 140 | 1 |
| 3 | 2 | 1 | 170 | 2 |
| 3 | 3 | 0 | 170 | 2 |
| 4 | 0 | 0 | 130 | 1 |
| 4 | 0 | 1 | 170 | 1 |
| 4 | 1 | 0 | 170 | 1 |
| 4 | 1 | 1 | 210 | 1 |
| 4 | 2 | 0 | 220 | 1 |
| 5 | 0 | 0 | 230 | 1 |
| 4 | 2 | 1 | 260 | 2 |
| 4 | 3 | 0 | 270 | 1 |
| 4 | 4 | 0 | 340 | 2 |
| 5 | 0 | 1 | 310 | 1 |
| 5 | 1 | 0 | 330 | 1 |
| 5 | 1 | 1 | 460 | 1 |
| 5 | 1 | 2 | 630 | 2 |
| 5 | 2 | 0 | 490 | 1 |
| 5 | 2 | 1 | 700 | 1 |
| 5 | 2 | 2 | 940 | 1 |
| 5 | 3 | 0 | 790 | 1 |
| 5 | 3 | 1 | 1100 | 1 |
| 5 | 3 | 2 | 1400 | 1 |
| 5 | 3 | 3 | 1700 [12] | 2 |
| 5 | 4 | 0 | 1300 | 1 |
| 5 | 4 | 1 | 1700 | 1 |
| 5 | 4 | 2 | 2200 | 1 |
| 5 | 4 | 3 | 2800 | 1 |
| 5 | 4 | 4 | 3500 | 2 |
| 5 | 5 | 0 | 2400 | 1 |
| 5 | 5 | 1 | 3500 | 1 |
| 5 | 5 | 2 | 5400 | 1 |
| 5 | 5 | 3 | 9200 | 1 |
| 5 | 5 | 4 | 16000 | 1 |
| 5 | 5 | 5 | >18000 [13] | 1 |

[11] 0 according to ISO 7218: Amd1. The use of <18 is common practice in bivalve mollusc sanitation programmes.
[12] 1800 according to ISO 7218: Amd1 but 1700 according to the ISO MPN calculator. 1700 confirmed by ISO working group on statistics for food microbiology.
[13] ∞ according to ISO 7218: Amd1. The use of >18000 is common practice in bivalve mollusc sanitation programmes.

## 12.2.2. Table 2: MPN table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 x 0.001g.

| 1g | 0.1g | 0.01g | 0.001g | MPN/100g [14] | Category |
|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | <18 | 1 |
| 0 | 1 | 0 | 0 | 18 | 1 |
| 1 | 0 | 0 | 0 | 20 | 1 |
| 1 | 0 | 1 | 0 | 40 | 2 |
| 1 | 1 | 0 | 0 | 40 | 1 |
| 1 | 2 | 0 | 0 | 61 | 2 |
| 2 | 0 | 0 | 0 | 45 | 1 |
| 2 | 0 | 1 | 0 | 68 | 2 |
| 2 | 1 | 0 | 0 | 68 | 1 |
| 2 | 1 | 1 | 0 | 92 | 2 |
| 2 | 2 | 0 | 0 | 93 | 1 |
| 3 | 0 | 0 | 0 | 78 | 1 |
| 3 | 0 | 1 | 0 | 110 | 1 |
| 3 | 1 | 0 | 0 | 110 | 1 |
| 3 | 1 | 1 | 0 | 140 | 2 |
| 3 | 2 | 0 | 0 | 140 | 1 |
| 3 | 2 | 1 | 0 | 170 | 2 |
| 3 | 3 | 0 | 0 | 170 | 2 |
| 4 | 0 | 0 | 0 | 130 | 1 |
| 4 | 0 | 1 | 0 | 170 | 1 |
| 4 | 1 | 0 | 0 | 170 | 1 |
| 4 | 1 | 1 | 0 | 210 | 1 |
| 4 | 2 | 0 | 0 | 220 | 1 |
| 5 | 0 | 0 | 0 | 230 | 1 |
| 4 | 2 | 1 | 0 | 260 | 2 |
| 4 | 3 | 0 | 0 | 270 | 1 |
| 4 | 4 | 0 | 0 | 330 | 2 |
| 5 | 0 | 1 | 0 | 310 | 1 |
| 5 | 1 | 0 | 0 | 330 | 1 |
| 5 | 1 | 0 | 1 | 450 | 2 |
| 5 | 1 | 1 | 0 | 450 | 1 |
| 5 | 1 | 2 | 0 | 620 | 2 |
| 5 | 2 | 0 | 0 | 490 | 1 |
| 5 | 2 | 0 | 1 | 690 | 2 |
| 5 | 2 | 1 | 0 | 690 | 1 |
| 5 | 2 | 1 | 1 | 920 | 2 |
| 5 | 2 | 2 | 0 | 930 | 1 |
| 5 | 3 | 0 | 0 | 780 | 1 |
| 5 | 3 | 0 | 1 | 1100 | 1 |
| 5 | 3 | 1 | 0 | 1100 | 1 |
| 5 | 3 | 1 | 1 | 1400 | 2 |
| 5 | 3 | 2 | 0 | 1400 | 1 |
| 5 | 3 | 2 | 1 | 1700 | 2 |
| 5 | 3 | 3 | 0 | 1700 | 2 |
| 5 | 4 | 0 | 0 | 1300 | 1 |
| 5 | 4 | 0 | 1 | 1700 | 1 |
| 5 | 4 | 1 | 0 | 1700 | 1 |
| 5 | 4 | 1 | 1 | 2100 | 1 |
| 5 | 4 | 2 | 0 | 2200 | 1 |
| 5 | 4 | 2 | 1 | 2600 | 2 |
| 5 | 4 | 3 | 0 | 2700 | 1 |
| 5 | 4 | 4 | 0 | 3400 | 2 |
| 5 | 5 | 0 | 0 | 2300 | 1 |

[14] See footnotes to Table 1.

## Table 2 continued: MPN table for multiple tube methods using 5 × 1g, 5 × 0.1g, 5 × 0.01g, 5 x 0.001g.

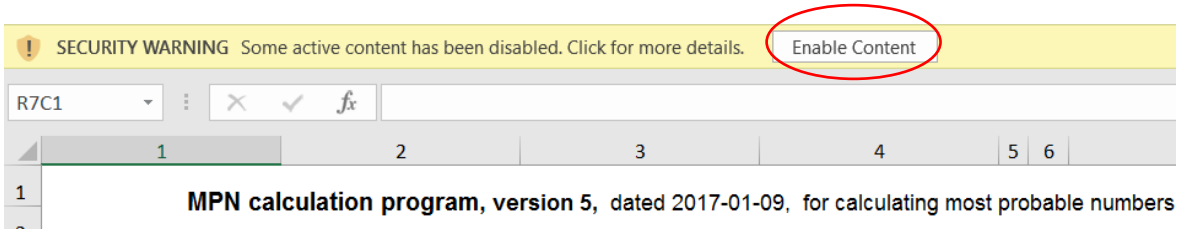
| 1g | 0.1g | 0.01g | 0.001g | MPN/100g | Category |
|---|---|---|---|---|---|
| 5 | 5 | 0 | 1 | 3100 | 1 |
| 5 | 5 | 1 | 0 | 3300 | 1 |
| 5 | 5 | 1 | 1 | 4600 | 1 |
| 5 | 5 | 1 | 2 | 6300 | 2 |
| 5 | 5 | 2 | 0 | 4900 | 1 |
| 5 | 5 | 2 | 1 | 7000 | 1 |
| 5 | 5 | 2 | 2 | 9400 | 1 |
| 5 | 5 | 3 | 0 | 7900 | 1 |
| 5 | 5 | 3 | 1 | 11000 | 1 |
| 5 | 5 | 3 | 2 | 14000 | 1 |
| 5 | 5 | 3 | 3 | 17000 | 2 |
| 5 | 5 | 4 | 0 | 13000 | 1 |
| 5 | 5 | 4 | 1 | 17000 | 1 |
| 5 | 5 | 4 | 2 | 22000 | 1 |
| 5 | 5 | 4 | 3 | 28000 | 1 |
| 5 | 5 | 4 | 4 | 35000 | 2 |
| 5 | 5 | 5 | 0 | 24000 | 1 |
| 5 | 5 | 5 | 1 | 35000 | 1 |
| 5 | 5 | 5 | 2 | 54000 | 1 |
| 5 | 5 | 5 | 3 | 92000 | 1 |
| 5 | 5 | 5 | 4 | 160000 | 1 |
| 5 | 5 | 5 | 5 | >180000 | 1 |

# 12.3. Appendix 3: Application and use of Excel MPN calculator (ISO 7218)

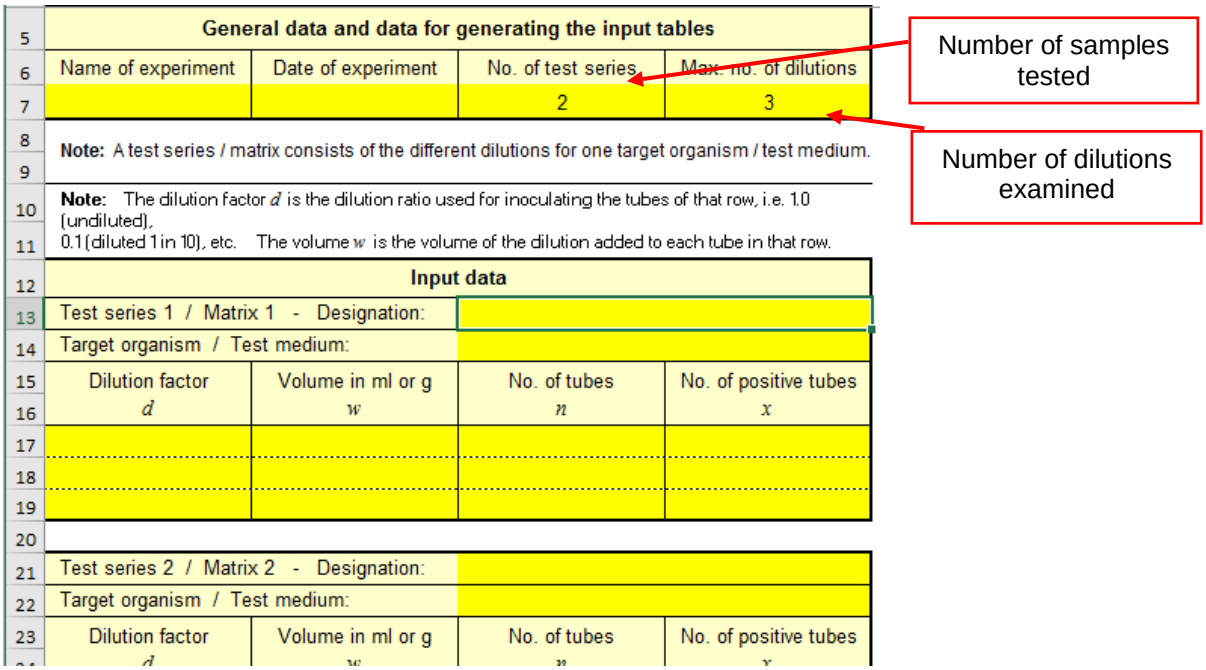
1. Open the link http://standards.iso.org/iso/7218/.

2. The ISO Standards Maintenance portal screen will appear. Click once the underlined name 'MPN calculator Excel programme ver5'.


3. On clicking the 'MPN calculator' link, an MPN calculator program excel spreadsheet will open. Click on the 'Enable Content' button at the top of the spreadsheet to enable all macros.




4. Enter relevant data in the general data section. The no. of test series (column 3, equivalent to number of samples, maximum of 30) and max. no. of dilutions cells (column 4) must be completed. Name of experiment and date of experiment cells (columns 1 and 2) do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files. Once the cells are filled, the required number of data boxes will automatically appear below (one for each test series/sample specified).




5. Populate the input data table as follows (test series designation and target organism cells do not need to be completed for the calculator to work but the user may wish to record these if saving completed calculator files):-

- Column 1 - Insert the homogenate concentration/dilution factor used to inoculate the MMGB tubes at each dilution (0.1 for the neat and $10^{-1}$ dilutions, 0.01 for the $10^{-2}$ dilution, 0.001 for

the $10^{-3}$ dilution if used).

- Column 2 - Insert the volume of inoculum added per tube at each dilution (10 for the neat dilution, 1 for all subsequent dilutions).
- Column 3 – Insert the number of MMGB tubes inoculated at each dilution (for the *E. coli* generic protocol 5 tubes are inoculated for each dilution).
- Column 4 – Insert the number of positive tubes (after confirmation using TBGA/TBX) at each dilution.

| Input data | | | |
|---|---|---|---|
| Test series 1 / Matrix 1 - Designation: | | Example 1 | |
| Target organism / Test medium: | | E.coli | |
| Dilution factor *d* | Volume in ml or g *w* | No. of tubes *n* | No. of positive tubes *x* |
| 0.1 | 10.0 | 5 | 5 |
| 0.1 | 1.0 | 5 | 4 |
| 0.01 | 1.0 | 5 | 2 |
| | | | |
| Test series 2 / Matrix 2 - Designation: | | Example 2 | |
| Target organism / Test medium: | | E.coli | |
| Dilution factor *d* | Volume in ml or g *w* | No. of tubes *n* | No. of positive tubes *x* |
| 0.1 | 10.0 | 5 | 4 |
| 0.1 | 1.0 | 5 | 3 |
| 0.01 | 1.0 | 5 | 1 |

Homogenate concentration used to inoculate MMGB

Volume of inoculum added

Number of MMGB tubes

Number of positive tubes on TBGA/TBX

6. Press 'Ctrl M' or press Calculate results (green button). The MPN value **in MPN per g** will be calculated in column 8 and the probability category for the combination shown in column 14.

| Results of the MPN calculations | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Test series / Matrix | | MPN | $\log_{10}$ MPN | SD $\log_{10}$ MPN | 95% confidence limits | | Rarity Index | Category |
| No. | Designation | | | | Lower | Upper | | |
| 1 | Example 1 | 22. | 1.3 | 0.20 | 8.8 | 56. | 0.304 | 1 |
| 2 | Example 2 | 3.3 | 0.51 | 0.18 | 1.4 | 7.5 | 0.010 | 3 |

Note: An MPN that falls into category 3 is unreliable and should not be used. The respective rows are marked in blue.

MPN/g

Category number

7. Check the probability category of each sample tested and accept only MPN results that give a category 1 or category 2 profile. Reject all MPN results show as probability category 3.

- For example 1 - the result (category 1) can be used.
- For example 2 - the result (category 3) is rejected and reported as a 'Void' result. A repeat sample must be requested.

8. The MPN calculator displays results in units of MPN per gram (MPN/g). *E. coli* results for shellfish must be reported as MPN per 100g (MPN/100g). Multiply the value given in column 8 by 100 to give the results in MPN per 100g.

For the examples given above

- For example 1 - 22 x 100 = 2200 MPN /100g.
- For example 1 - A repeat sample would need to be requested as it has a category number of 3.

9. For samples that give a tube combinations of 0, 0, 0 and 5, 5, 5 (3 dilutions) or 0, 0, 0, 0 and 5, 5, 5, 5 (4 dilutions) the MPN calculator will provide a value of 0 and infinity respectively (see below). As explained in section 8.9 results for these tube combinations should be reported as <18 (0, 0 ,0 or 0, 0, 0, 0), >18000 (5, 5, 5) and >180000 MPN per 100g (5, 5, 5, 5) respectively.

| data | |
|---|---|
| Example 1 | |
| E.coli | |
| No. of tubes *n* | No. of positive tubes *x* |
| 5 | 0 |
| 5 | 0 |
| 5 | 0 |

| Example 2 | |
|---|---|
| E.coli | |
| No. of tubes *n* | No. of positive tubes *x* |
| 5 | 5 |
| 5 | 5 |
| 5 | 5 |

**Results of the MPN calculations**

| No. | Test series / Matrix Designation | MPN | $\log_{10}$ MPN | SD $\log_{10}$ MPN | 95% confidence limits Lower | 95% confidence limits Upper | Rarity Index | Category |
|---|---|---|---|---|---|---|---|---|
| 1 | Example 1 | 0 | | | 0 | 0.66 | 1.000 | 1 |
| 2 | Example 2 | ∞ | | | 65. | ∞ | 1.000 | 1 |

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