

NATIONAL STANDARD METHOD

# DETECTION OF *SALMONELLA* SPECIES

W 7

Issued by Standards Unit, Evaluations and Standards Laboratory  
Centre for Infections

**DETECTION OF *SALMONELLA* SPECIES**

Issue no: 3 Issue date: 09.07.06 Issued by Standards Unit, Evaluations and Standards Laboratory, in conjunction with the  
Regional Food, Water and Environmental Coordinators Forum. Page 1 of 16

Reference no: W 7i3

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## Suggested citation for this document:

Health Protection Agency (2006). *Detection of Salmonella species*. National Standard Method W 7 Issue 3. [http://www.hpa-standardmethods.org.uk/pdf\\_sops.asp](http://www.hpa-standardmethods.org.uk/pdf_sops.asp).

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# AMENDMENT PROCEDURE

<b>Controlled document reference</b>	<b>W 7</b>
<b>Controlled document title</b>	<b>Standard Operating Procedure for Detection of <i>Salmonella</i> species</b>

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from [standards@hpa.org.uk](mailto:standards@hpa.org.uk).

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
6/ 09.07.06	2.3	3	5	<b>Background</b>	Amended to include use of MKTTn and use of selenite cystine broth in certain cases
			6	<b>Principle &amp; Safety Considerations</b>	Updated
			7	<b>4 Equipment</b>	Thick glass fibre added
			8	<b>5 Culture Media and Reagents</b>	Amended to include use of MKTTn in line with ISO 19250  Supplementary isolation agar added  Optional in-house and biochemical test replaced with API 20E system updated
			10	<b>6.3 Recognition of colonies</b>	Updated to include API 20E system
			11	<b>6.4 Confirmatory tests</b>	Updated to include Salmonella Group
			12	<b>7 Reporting</b>	Updated
			12	<b>9 Reference facilities</b>	Added
			13	<b>Acknowledgments and contacts</b>	Updated
			14	<b>Appendix</b>	Updated
			15	<b>References</b>	Updated

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# STANDARD OPERATING PROCEDURE FOR THE DETECTION OF *SALMONELLA* SPECIES

## INTRODUCTION

### SCOPE

The method described is applicable to the isolation and detection of *Salmonella* species from all types of water. *Salmonella* species are concentrated by membrane filtration (non-turbid waters) or a filter pad with filter aid (turbid waters).

### BACKGROUND

Surface waters and reservoirs are particularly liable to pollution from animals and birds, and *Salmonella* species may be detected when only small numbers of indicator organisms are present eg *Escherichia coli*. For technical reasons, detection tests for *Salmonella* species are not routinely carried out on all samples. However, potable waters may be examined as part of epidemiological investigations into the source of enteric infections, and river or recreational waters may be examined as part of local authority surveillance programmes. Coastal or inland waters as directed by the Bathing Waters (Classification) Regulations 1991<sup>2</sup> may also be examined at specified intervals for *Salmonella* species.

This method is based on the method described in the Draft ISO 19250<sup>3</sup>. This uses Rappaport Vassiliadis soya peptone (RVS) broth, which is highly effective for recovery of *Salmonella* from water with a high level of background contamination. For toxicological reasons it also replaces the use of a selenite medium by Muller Kauffmann tetrathionate novobiocin broth (MKTTn) for the isolation of serotypes of *Salmonella* that are inhibited by the constituents of RVS broth. Although extensive trials have been performed to demonstrate the efficacy of these media for the recovery of *Salmonella* Typhi<sup>4,5</sup> and *Salmonella* Paratyphi<sup>4,5</sup>, it is recognised that the combination of these two selective media may not allow recovery of all these strains. This method therefore retains the use of selenite cystine (SC) broth<sup>6</sup> for samples in which *Salmonella* Typhi and *Salmonella* Paratyphi are specifically sought.

Two isolation media are specified; these are Xylose Lysine Deoxycholate (XLD) agar and Brilliant Green agar (BGA). If *Salmonella* Typhi or *Salmonella* Paratyphi are specifically sought these isolation media should be supplemented with another selective plating medium that does not contain a high concentration of brilliant green, as this may inhibit the recovery of these strains.

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# 1 DEFINITIONS

For the purposes of this method the following definitions apply:

## *Salmonella* species

Microorganisms which form characteristic colonies on selective agar media and which produce the serological and biochemical reactions described in this method.

## *Detection of Salmonella* species

Determination of the presence or absence of these microorganisms in a 1 litre or other specified volume when tests are carried out in accordance with this method.

# 2 PRINCIPLE

The detection of *Salmonella* species in water involves concentration of organisms by passage of water through a membrane filter(s) or water mixed with filter aid is passed through a pad<sup>7</sup>. The membrane filter(s) or pad containing filter aid are incubated in a non-selective liquid pre-enrichment medium followed by enrichment in selective liquid media, subculture onto each of two different selective solid media and examination for colonies considered to be typical of *Salmonella* species. Confirmation of colonies as *Salmonella* species is by means of serological and biochemical tests.

# 3 SAFETY CONSIDERATIONS<sup>8-17</sup>

Normal microbiology laboratory precautions apply.

*Salmonella* Typhi and *Salmonella* Paratyphi are classified as **Hazard Group 3** organisms and must be handled under **Containment Level (CL) 3** conditions.

Note: If samples are linked to an outbreak where either *Salmonella* Typhi or *Salmonella* Paratyphi is a suspected cause, all samples should be handled under CL 3 conditions.

## 3.1 SAMPLE COLLECTION

Samples should be taken in accordance with the Standing Committee of Analysts part 2<sup>18</sup>.

## 3.2 SAMPLE TRANSPORT AND STORAGE

Compliance with current transportation regulations is essential. Transport in accordance with the Standing Committee of Analysts part 2<sup>18</sup>.

## 3.3 SAMPLE PROCESSING

- Salmonellas are pathogenic to man and therefore isolation and identification must be carried out by trained laboratory personnel in a properly equipped laboratory and under the supervision of a qualified microbiologist
- Care must be taken in the disposal and sterilisation of all test materials
- Procedures involving subculturing of pre-enrichment and enrichment broths and handling of cultures of *Salmonella* species must be carried out in a designated laboratory or part of a laboratory
- Subculture and identification procedures for *Salmonella* Typhi and *Salmonella* Paratyphi must be carried out in a CL 3 laboratory and disposable gloves should be worn during all procedures
- Selenium salts are used in the preparation of selenite cystine broth. They are toxic if ingested or inhaled and there is a possible risk of teratogenicity in pregnant laboratory workers

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**The above guidance should be supplemented with local COSHH and risk assessments and CL3 working policies**

## 4 EQUIPMENT

Usual laboratory equipment and in addition:

- Membrane filtration manifold
- Filter funnels (graduated)
- Pyrex vacuum flask with protective jacket: large volume eg 5 L or alternative
- Vacuum pump with moisture trap or protective filter or alternative vacuum source
- Stainless steel flat tipped forceps
- Boiling waterbath (instrument steriliser)
- Incubators: 37°C±1°C 41.5°C±1°C
- Sterile absorbent pads
- Thick glass fibre pre-filter (40 mm)
- Cellulose ester 0.45 µm pore size gridded membrane filters
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 1 mL and 0.1 mL volumes (optional)
- Pipettes (sterile total delivery) 1 mL graduated in 0.1 mL volumes (optional)

**Note:** Care must be taken to avoid cross contamination during the use of automatic pipettors<sup>19</sup>.

## 5 CULTURE MEDIA AND REAGENTS

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

### *Filter aid*

Hyflo – supercel	1.0 g
Water	15 mL

### *Buffered peptone water (BPW)*

Enzymatic digest of casein	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate dodecahydrate	9.0 g
Potassium dihydrogen phosphate	1.5 g
Water	1 L
pH 7.2 ± 0.2 at 25°C	

### *Selenite cystine broth (SC) for Salmonella Typhi and Salmonella Paratyphi only*

Tryptone	5.0 g
Lactose	4.0 g
Disodium phosphate	10.0 g
L-Cystine	10 mg
Sodium biselenite	4.0 g
Water	1 L
pH 7.0 ± 0.2 at 25°C	

### *Rappaport Vassiliadis soya peptone broth (RVS)*

Soya peptone	4.5 g
Sodium chloride	7.2 g

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Potassium dihydrogen phosphate	1.26 g
Di-potassium hydrogen phosphate	0.18 g
Magnesium chloride (anhydrous)	13.58 g
Malachite green	36 mg
Water	1 L
pH 5.2 ± 0.2 at 25°C	

*Muller-Kauffmann tetrathionate novobiocin broth (MKTTn)*

Meat extract	4.3 g
Enzymatic digest of casein	8.6 g
Sodium chloride	2.6 g
Calcium carbonate	38.7 g
Sodium thiosulphate pentahydrate	47.8 g
Ox bile	4.78 g
Brilliant green	9.6 mg
Iodine	4.0 g
Potassium iodide	5.0 g
Novobiocin (sodium salt)	0.04 g
Water	1 L
pH (of basal broth) 8.0 ± 0.2 at 25°C	

*Xylose lysine desoxycholate agar (XLD)*

Yeast extract	3.0 g
L-lysine hydrochloride	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium desoxycholate	1.0 g
Sodium chloride	5.0 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	0.8 g
Phenol red	80 mg
Agar	12.5 g
Water	1 L
pH 7.4 ± 0.2 at 25°C	

*Brilliant Green agar (modified) (BGA)*

Meat extract powder	5.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Disodium hydrogen phosphate	1.0 g
Sodium dihydrogen phosphate	0.6 g
Lactose	10.0 g
Sucrose	10.0 g
Phenol red	0.09 g
Brilliant green	4.7 mg
Agar	12.0 g
Water	1 L
pH 7.0 ± 0.2 at 25°C	

*Supplementary isolation agar of choice (eg: Hynes DCA, Rambach agar) (optional)*

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*MacConkey agar (MA) (or similar)*

Bile salts	5.0 g
Peptone	20.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Neutral red	50 mg
Agar	12.0 g
Water	1 L
pH 7.4 ± 0.2 at 25°C	

*Cystine lactose electrolyte deficient agar (CLED)*

Peptone	4.0 g
Meat extract	3.0 g
Tryptone	4.0 g
Lactose	10.0 g
L-cystine	0.128 g
Bromothymol blue	20 mg
Agar	15.0 g
Water	1 L
pH 7.3 ± 0.2 at 25°C	

*Slopes of nutrient agar (NA), Columbia agar (or equivalent)*

*Saline solution*

Sodium chloride	8.5 g
Water	1 L

*Serological reagents for identification of Salmonella species*

*API 20E identification system and appropriate API reagents*

## 6 SAMPLE PROCESSING

### 6.1 SAMPLE PREPARATION AND DILUTIONS

Water samples should be received and handled as described in SOP W 1, Section 5. The nature of the request and condition of the sample should be noted on arrival. Protect the samples from direct sunlight and transport in an insulated container or refrigerator at 2°C – 8°C. Samples should be analysed as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

Following the procedures laid down in SOP W 1, Section 5 select suitable volumes for analysis.

### 6.2 FILTRATION AND INCUBATION

*Membrane filtration (non-turbid waters)*

Filter 1 L of water following the procedures laid down in SOP W 1, Section 5.

At the end of filtration use flat-ended forceps to remove the filter from the holder and transfer directly into a minimum of 90 mL BPW. Where more than one filter has been used then culture the filters collectively.

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#### *Filter aid (turbid waters)*

The use of filter aid is recommended for samples that are too turbid for membrane filtration.

Place a sterile absorbent pad on the membrane filtration apparatus and filter 15 mL of well mixed sterile filter aid suspension to give a layer of filter aid on the pad. Next, add 15 mL of well mixed filter aid to 1 L of water. Mix well and filter. After filtration, remove the pad and filter aid and transfer directly to 90 mL BPW. Rinse any filter aid adhering to the funnel into the container and mix well with the medium.

#### *Pre-enrichment*

Place the inoculated BPW in an incubator at 37°C ± 1°C for 18 hours ± 2 hours.

#### *Selective enrichment*

Transfer 0.1 mL of the pre-enrichment culture to 10 mL of RVS. Place in an incubator at 41.5°C for 24 ± 3 h and subculture to selective agars. Re-incubate and repeat if necessary.

Transfer 1 mL of the pre-enrichment culture to 10 mL of MKTTn. Place in an incubator at 37°C for 24 ± 3 h.

If typhoid or paratyphoid is suspected, transfer 1 mL of the same pre-enrichment culture to 10 mL of SC broth. Place in an incubator at 37°C ± 1°C, for 24 hours ± 3 hours and subculture to selective agars.

If *Salmonella* Typhi or *Salmonella* Paratyphi are sought, also subculture to a further isolation medium that does not contain high levels of brilliant green eg: Hynes DCA.

Note: These operations should be carried out under CL 3 conditions.

In certain circumstances, such as outbreak investigations and surveys, it may be necessary to re-incubate the RVS and SC broths for a further 24 hours and make additional subcultures to selective agars.

#### *Subculture to selective agars*

Following incubation subculture the selective enrichment broths to XLD and to BGA. Subculture all broths after 24 ± 3 h and again after 48 ± 3 h if the broth was re-incubated. Place in an incubator at 37°C for 24 ± 3 h.

### **6.3 RECOGNITION OF COLONIES**

After 24 h examine the selective agar plates for typical and atypical colonies of *Salmonella*.

On XLD, *Salmonella* ferment xylose, normally decarboxylate lysine, and produce hydrogen sulphide. Characteristic colonies are red with black centres. Isolated colonies may appear yellow with black centres. *Salmonella* species that produce little or no hydrogen sulphide eg. *Salmonella* Typhi, *Salmonella* Senftenberg, *Salmonella* Pullorum grow as red colonies with or without black centres. Red colonies may also be produced by some strains of *Proteus* species and *Pseudomonas* species. Strains of *Salmonella* Paratyphi A do not decarboxylate lysine and so appear as yellow colonies usually with a black centre. Lactose fermenting strains may also appear yellow with or without black centres.

Note: All strains of *Salmonella* Typhi and *Salmonella* Paratyphi other than *Salmonella* Paratyphi A are lysine positive. Production of black colonies due to hydrogen sulphide on XLD is variable. *Salmonella* Typhi may not grow on XLD.

On BGA, *Salmonella* species do not normally ferment sucrose or lactose and produce red colonies surrounded by a bright red medium. Red colonies may also be produced by some strains of *Proteus* species and *Pseudomonas* species. Strains of *Salmonella* Typhi and *Salmonella* Paratyphi may not grow on BGA.

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## 6.4 CONFIRMATORY TESTS

Typical or suspect colonies of *Salmonella* from each plate must be subjected to serological and biochemical confirmation. The extent of testing may be reduced once the presence of *Salmonella* is established (see below).

In some instances a rapid response may be required. Provisional recognition of *Salmonella* can be obtained by performing agglutination with polyvalent 'O' and 'H' antisera on discrete colonies obtained on the primary isolation media. Alternatively NA slopes may be inoculated directly from the primary isolation agar but a purity check on MA or CLED must also be inoculated after inoculation of the slopes.

Select at least five suspect *Salmonella* colonies including one from each of the selective agar plate / broth combinations and inoculate purity plates by subculturing to MA or CLED agar. Place in an incubator at 37°C for 21 ± 3 h.

### *Serological confirmation*

Subculture non - lactose fermenting colonies from the above media to NA or equivalent slopes dispensed in universal containers. Ensure that some water of condensation is present at the base of the slope; if none is present then add a few drops of sterile water. Inoculate the colony into the water of condensation and streak up the slope. Incubate at 37°C for 5 ± 1 h, or overnight if necessary.

Using the growth from the NA slope prepare three saline suspensions on a slide using a loopful of saline and growth from: -

- the slope for 'O' antigens
- the water of condensation at the bottom of the slope for 'H' antigens
- a mixture from slope and condensate for autoagglutination. If autoagglutination occurs proceed to biochemical confirmation

Add a loopful of polyvalent 'O' and polyvalent 'H' antisera to two separate saline suspensions and rock the slide gently for 30 seconds. If agglutination occurs with the polyvalent antisera but not with the saline the reactions are considered to be positive. The test is considered to be equivocal if agglutination occurs with saline (autoagglutination).

Identify to at least *Salmonella* group level by agglutination with specific 'O' antisera. If possible also identify at least one 'H' antigen. Record the results.

The first isolate must be serologically typed as comprehensively as possible, determining the 'O' and 'H' antigens. All subsequent isolates must be serotyped to determine the 'O' antigen and one 'H' antigen only. If the slide agglutination reactions indicate that subsequent isolates differ serologically from the first isolate then full serological and biochemical testing must be performed.

Note: Some *Salmonella* species may give agglutination with 'H' antisera but not with 'O' antisera. This could be due to the presence of antigens not included in the polyvalent 'O' antiserum or to the masking of 'O' antigens by capsular antigens. Such isolates must either be tested with individual 'O' antisera or Vi antiserum if *Salmonella* Typhi is suspected.

### *Biochemical confirmation*

Select colonies from the MA or CLED agar purity plates and inoculate biochemical tests.

Identify at least one isolate giving biochemical and/or serological reactions consistent with *Salmonella* or those giving equivocal serological results using API 20E identification system.

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The API 20E identification system must be inoculated, incubated and read as described in the manufacturers instructions. Identification is obtained by using the resulting numerical profile and utilising the up to date database and identification system available on the Biomerieux website <https://apiweb.biomerieux.com>.

**Note:** If more than one serotype is present then a representative of each serotype must also be identified biochemically.

## 7 REPORTING

Report the results using the procedure described in SOP W 1 Section 9.

If *Salmonella* species are not isolated report as follows:

**'Salmonella species not detected in 1 litre of water'**

If *Salmonella* species are isolated report as follows:

either

**'Salmonella species detected in 1 litre of water'**

or

**'Salmonella Group..... detected in 1 litre of water'**

Further identification to follow

In all instances, the amount of water examined must be reported. If 1 litre has not been examined the volume tested must be indicated on the report form.

## 8 QUALITY CONTROL

### *Membrane filtration*

When the membrane filtration technique is used internal quality control procedures must be carried out at least once a month depending on the workload of the laboratory. If more than one batch of media is used in a session it is necessary to repeat the quality control test for each batch.

The internal quality controls should be carried out using suspensions of positive and negative control organisms known to contain less than 100 colony forming units in the volume filtered.

### **Control cultures**

Positive control: *Salmonella* Nottingham (16:d:enz15) NCTC 7832

Negative control: *Escherichia coli* NCTC 9001

Blank control

Filter 1 L of sterile distilled water, peptone saline diluent or quarter strength Ringer's solution using the same funnel as that used for the positive control following sterilisation.

Incubate all tests and perform all procedures in parallel with the routine test samples.

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## 9 REFERENCE FACILITIES

All *Salmonella* isolates of public health significance must be referred to the Laboratory of Enteric Pathogens (LEP), Centre for Infections, HPA, Colindale for identification, confirmation and phage typing. Five colony picks (in total) of each colony type should be sent.

## 10 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the Water Working Group for Standard Methods ([http://www.hpa-standardmethods.org.uk/wg\\_water.asp](http://www.hpa-standardmethods.org.uk/wg_water.asp)). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

The National Standard Methods are issued by Standards Unit, Evaluations and Standards Laboratory, Centre for Infections, Health Protection Agency London.

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### DETECTION OF *SALMONELLA* SPECIES

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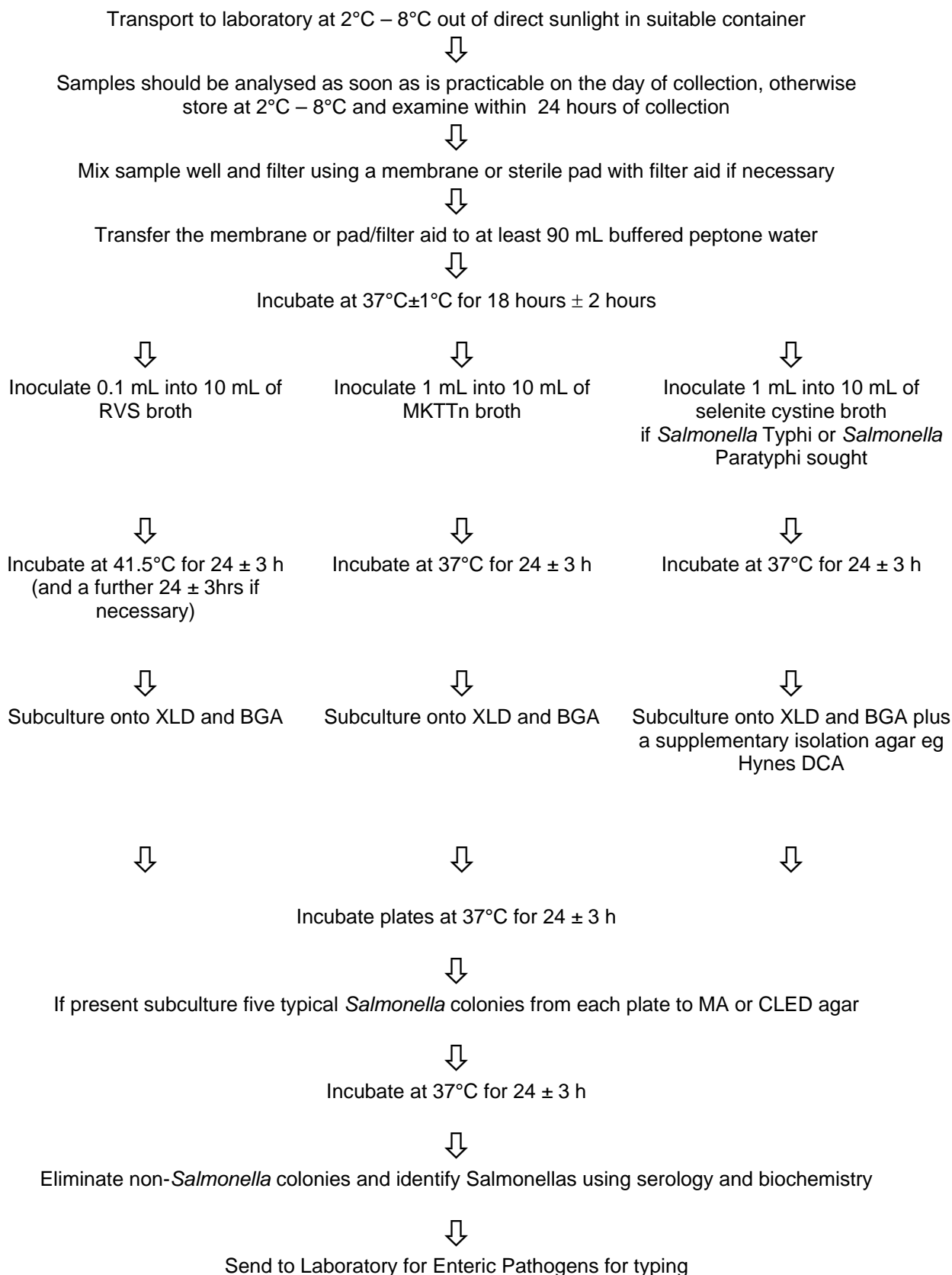
Reference no: W 7i3

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# APPENDIX: FLOWCHART SHOWING THE PROCESS FOR THE DETECTION OF *SALMONELLA* SPECIES



## DETECTION OF *SALMONELLA* SPECIES

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