APPENDIX

WHO Global Foodborne Infections Network

(formerly WHO Global Salm-Surv)

"A WHO network building capacity to detect, control and prevent foodborne and other enteric infections from farm to table"

Laboratory Protocol
"Isolation of *Salmonella* spp. From Food and Animal Faeces "
5th Ed. June 2010

IMPORTANT NOTES:

1) This procedure is based on the ISO protocol: 6579:2002 "Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Salmonella* spp."4. This protocol is intended to provide guidance for the testing of suspect food items/ animal faecal specimens identified via foodborne disease surveillance programmes. Regulatory agencies (Ministries of Health, Agriculture, Commerce, etc) have specific testing requirements, different from this protocol, which much be used to test samples collected for regulatory testing (example: import/export or product recall). Prior to performing any official, legal, or regulatory testing, the reader should confirm the appropriate protocol through consult with in-country regulatory authorities.

2) This protocol is intended only to be used on food samples and animal faeces. This protocol should not be used for the testing of human faeces.

Foreword:

Infections due to *Salmonella* spp. remain a global problem. These infections may cause significant morbidity and mortality both in humans and production animals as well as considerable economic losses. *Salmonella* spp. are typically transmitted among humans and animals via a fecal-oral route, usually through the consumption of contaminated food or water. Timely identification and serotyping of *Salmonella* from clinical specimens facilitates outbreak detection and patient management while prompt and accurate detection of *Salmonella spp.* in contaminated food or water provides an opportunity to prevent the contaminated food from entering the food supply.

Sensitive and specific laboratory methods for the isolation, identification, and serotyping of *Salmonella* are essential elements of *Salmonella* monitoring and control programmes. An ideal method will be rapid, inexpensive, easily reproducible, sensitive, and specific. Currently, no single method meets all these criteria and the optimal method may vary depending on the source of specimen (e.g., human clinical specimens, different food matrices, and environmental specimens) and the target serotype (e.g. typhoidal versus non-typhoidal *Salmonellae*). Additionally, new methods are being described regularly and comparison of current methodologies to new methodologies is highly recommended. To insure continuity of results, any new method must be validated and standardized prior to implementation.

The following protocol is based on the ISO-6579 standard method for the isolation and identification of *Salmonella* from food and animal faeces. This procedure has been extensively documented in the peer-reviewed literature and has been accepted by international accreditation agencies. It can be tailored to fit the needs of most laboratories world-wide. Molecular methodologies have the potential to increase sample throughput, sensitivity, and specificity, while simultaneously reducing turnaround time. However, it is important to utilize the appropriate test for the

appropriate sample, for example PFGE can provide supplementary discrimination below the level of serotype, however it should not take the place of serotyping. Also, many molecular assays are non-culture tests (or only require pre-enrichment). While these assays may provide rapid screening results, it is essential that an attempt be made to obtain an isolate for additional studies such as serotyping and susceptibility testing.

Detailed identification and serotyping procedures may be found in the Global Salm-Surv Laboratory Protocol "Identification of *Salmonella* and *Shigella* Using an Abbreviated Panel of Tests"7 and "Serotyping of *Salmonella*" and a flow diagram providing a brief overview of the isolation, identification, and serotyping procedure may be found in Appendix 3.

1. Isolation, identification and serotyping of *Salmonella* from faeces and food

Introduction:

The following procedures will guide you through the steps necessary to isolate *Salmonella* from animal faeces or food.

Description of Genus[2,7]:

The genus *Salmonella* is comprised of two species *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies which are designated by name or Roman numeral:

***Salmonella enterica* subspecies**	
I	*Salmonella enterica* subsp. *enterica*
II	*Salmonella enterica* subsp. *salamae*
IIIa	*Salmonella enterica* subsp. *arizonae*
IIIb	*Salmonella enterica* subsp. *diarizonae*
IV	*Salmonella enterica* subsp. *houtenae*
VI	*Salmonella enterica* subsp. *indica*

The majority of human *Salmonella* infections are caused by *S. enterica* subspecies I. Additionally, several invasive *Salmonella* serotypes; Typhi, Paratyphi A, Choleraesuis, Dublin, and Paratyphi C are all *S. enterica* subspecies I serotypes.

Salmonella serotype is determined by the immunoreactivity of three surface antigens "O" (LPS), "H" (flagellin protein), and "Vi" (capsule). Between the two species of *Salmonella*, over 2,500 unique serotypes have been described and new serotypes are described regularly.

As is typical of all other *Enterobacteriaceae*, the *Salmonellae* are Gram-negative, oxidase negative, facultative anaerobes. The *Salmonellae* are Vogues-Proskauer (VP) negative, methyl red positive, and reduce nitrate to nitrite without the production of gas. The *Salmonellae* are typically indol and urease negative, although rare indol or urease positive strains may be encountered. *Salmonellae* are typically motile by means of peritrichious flagella; however, nonmotile variants may be encountered and the host-adapted avian pathogens *Salmonella* serotypes Pullorum and Gallinarum are always nonmotile. There is considerable phenotypic variation between the two species of *Salmonella* and the six subspecies of *S. enterica* which may be utilized for differentiation. Additionally, several serotypes (notably *Salmonella* serotypes Typhi, Paratyphi A, Choleraesuis, and Paratyphi C) have biochemical profiles which are

unique from other *Enterobacteriaceae* and can be utilized to make a serotype level identification even in the absence of serology.

Isolation of *Salmonella* from Animal Faeces and Food[3,4,5,6]:

The isolation of *Salmonella* from animal feces may be complicated by several factors. Animals may be subclincally infected (i.e. not showing clinical signs of disease) and shedding small numbers of *Salmonellae* in their faeces. Additionally, the population of *Salmonellae* in feces is typically much lower than that of other enteric flora. Similarly, *Salmonella* populations in food samples may be stressed due to heat, pH, or salt content, or unevenly distributed through the food matrix. Several steps are taken to insure optimal recovery of *Salmonellae* from these samples:

1) Use a large sample volume (25g). This helps to insure accurate representation of the entire matrix.
2) Use a pre-enrichment step, such as growth in buffered peptone water before direct plating for Salmonella. This allows stressed or injured Salmonellae to recover before exposure to selective enrichment media.
3) Use selective media to preferentially recover Salmonella.
    a. Use Mueller-Kauffmann's Tetrathionate broth (TTmk) and Rappaport-Vassiliadis Soya Peptone broth (RVS) for pre-enrichment. TTmk provides good control of Escherichia while RVS provides good control of Proteus and Pseudomonads.
    b. Use Xylose-Lysine Desoxycholate agar (XLD) and Brilliant Green agar (BGA) for direct plating.

The protocol presented here is based on the ISO-6579 standard and is intended for use with animal faeces and food products intended for consumption by humans or production animals. It should be noted that the media used in this protocol are highly selective and may be inhibitory to some typhoidal Salmonellae (particularly Salmonella serotypes Typhi and Paratyphi A). Salmonella serotypes Typhi and Paratyphi A are host adapted human pathogens, under normal circumstances these serotypes are not be found in animal faeces and are infrequently implicated in foodborne outbreaks. However, if testing of food samples for Salmonella Typhi or Paratyphi A is performed, it is essential to supplement this procedure with both a selective enrichment broth and selective plating media which do not inhibit these serotypes (e.g. selenite-cysteine broth and desoxycholate agar or bismuth sulphate agar)[3].

Safety[1]:
Several countries follow the CDC/NIH biosafety recommendations published in the "Biosafety in Microbiological and Biomedical Laboratories" 5th Ed (BMBL-5) (check year). The BMBL recommends BSL-2 practices and procedures when working with serotypes other than Salmonella Typhi.
BSL-2 procedures are recommended for the routine manipulation (e.g. processing clinical samples) of Salmonella Typhi and BSL-3 practices and procedures are recommended when working with production quantities of Salmonella Typhi or performing procedures likely to generate aerosols.

References

1) Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Ed. 2007. Centers for Disease Control and Prevention, Atlanta, GA. Available at: http://www.cdc.gov/biosafety/publications/BMBL_5th_Edition.pdf

2) Brenner, F.W., & A.C. McWhorter-Murlin. 1998. Identification and Serotyping of *Salmonella*. Centers for Disease Control and Prevention, Atlanta, GA.

3) Health Protection Agency (2007). Detection of *Salmonella* species. National Standard Method F 13 Issue 3. http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

4) ISO-6579 : 2002 (E) 4th Ed. Microbiology- General Guidance on Methods for the detection of *Salmonella*, International Organisation for Standardization, Geneve, Switzerland.

5) NMKL method no. 71, 2nd ed., 1999: Salmonella. Detection in food. Nordic committee on food analysis.

6) Post D. E. (1997) Food-borne pathogens monograph number I *Salmonella*. Oxoid limited, Hampshire, England.

7) WHO Global Foodborne Infections Network. Laboratory Protocol: "Biochemical Identification of *Salmonella/Shigella* Using an Abbreviated Panel of Tests" January 2010. Available at: http://www.antimicrobialresistance.dk/data/images/protocols/gfn_biochem_final.pdf

1.1 Isolation of *Salmonella* from food and animal faeces.

Materials

Equipment :
Erlenmeyer flasks (500 ml) etc. sterile (for pre-enrichment)
Disposable inoculation loops (1 µl and 10 µl)
Plastic petri dishes (9 cm diameter) sterile
Balance
Incubators at 37ºC and 41.5ºC
Bunsen burner
Pipettes for 0.1 ml (e.g. 1 ml pipettes)
Wood spatulas

Media:
Buffered peptone water 225 ml
Tetrathionate broth (Müller-Kauffmann) 10 ml
Rappaport Vassiliadis soy peptone broth 10 ml
Xylose Lysine Desoxycholate (XLD) agar plates
Brilliant Green (BGA) agar plates
Nutrient agar plates

Bacterial strains
Food samples
Animal Faecal samples

Safety
Carry out all procedures in accordance with the local codes of safe practice.

Specimen Collection and Transport:
Samples may consist of food or animal faeces.
Ideally, at least 25g of food or animal faeces should be submitted. However, smaller samples may be submitted if larger samples cannot be obtained.
Salmonella spp. may not be evenly distributed within a sample. Specimens should be mixed prior to testing and specimens should be obtained from several locations within the sample.
Food samples should be transported to the laboratory at the appropriate temperature. Foods should be maintained at their recommended storage temperature during transport: Frozen foods (example: ice cream) should be remain frozen for transport; cold foods (example: milk) should be kept cold (not frozen) for transport; and room

temperature foods (example: powdered formula) should be transported at room temperature.

Faecal samples must be submitted in a clean, container with no soap or disinfectant residue. Small faecal samples (example: swabs from small animals) may be placed in transport media. The sample must be kept cold and transported to the lab within 8 hours of collection. If the sample cannot reach the laboratory within 8 hours; the sample should be frozen at < 70°C or stored on dry ice.

Procedure:

Day 1: Non-selective pre-enrichment

Weigh out 25 g food or animal faeces with a sterile wood spatula, place the sample into an Erlenmeyer flask and add 225 ml buffered peptone water to obtain 1 part sample + 9 part buffer. Mix. Incubate at 36°C (+/- 1°C) overnight (16-20 hours).

Day 2: Prepare selective enrichment (I) and (II)

Use a pipette to transfer 1 ml of the pre-enrichment broth to 10 ml Tetrathionate broth (Müller-Kaufmann). (Label as Tube I)

Use a micro-pipette to transfer 0.1 ml (100 uL) of the pre-enrichment broth to 10 ml Rappaport-Vassiliadis soy peptone (RVS) broth. (Label as Tube II)

Incubate Tube I: Tetrathionate broth (Müller-Kaufmann) at 36.0°C ± 1°C and Tube II: Rappaport-Vassiliadis soy peptone (RVS) at 41.5°C± 0.5°C overnight (18-24 hours).

Day 3: Spread on selective agar plates

Spread a 10 µl loop full from the inoculated and incubated Tetrathionate broth (I) and RVS broth (II) on XLD and on BGA agar plates and incubate at 36.0°C ± 1°C overnight (18-24 hours).

Day 4: Selection and Subculture of Suspect *Salmonella* Colonies

Examine the XLD plates:

A typical *Salmonella* colony has a slightly transparent red halo and a black centre, a pink-red zone may be seen in the media surrounding the colonies. Note the presence of typical *Salmonella*- like colonies on XLD with a + in the record sheets.

Examine the BGA plates:

Typical *Salmonella* colonies on a BGA agar plate appear red and impart a red/pink colour to the surrounding agar. Other enterics typically appear green or yellow. Note the presence of typical ***Salmonella*-like colines on BGA with a + in the record** sheets. Plate two suspect colonies from XLD agar and BGA onto non-selective media (e.g. nutrient agar) for biochemical confirmation and serotyping.

Day 5-7: Biochemical Identification and Serotyping:

Please refer to WHO GFN Procedures "Identification of *Salmonella* and *Shigella* using an Abbreviated Panel of Tests" and "Serotyping of *Salmonella*".

Theory / Comments:

The volume of the food or faeces sampled determines the sensitivity of detection. Sensitivity increases with larger sample volumes. In practice, however, laboratories may receive samples smaller or larger than 25g. While not ideal, it is not uncommon for swabs (volume less than 1 gram) to be submitted. Or to receive large samples in excess of 25 grams.

It is critical that the ratio of 1 part sample plus 9 part buffered peptone water be maintained. When small samples are received, the volume of buffered peptone water must be decreased. To maintain the ratio of 1 part sample plus 9 parts buffered peptone water.

With large samples, the sample should be completely mixed and a 25g sample should be obtained by collecting small portions from various areas of the sample. Alternatively, the sample can be divided into 25g segments and inoculated into 2 or more bottles of buffered peptone water.

This ratio also applies to pooled samples, for instance 5x5 grams of faeces to 225 ml pre-enrichment broth.

2. Composition and preparation of culture media and reagents

If no reference is given, it is the procedure used at DVL.
The media and reagents are available from several companies including Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturers description if it differs from the description given here. Refer to Appendix 2 for a colour presentation of growth of *Salmonella* on selective agar media and positive and negative reactions of biochemical tests.

Brilliant Green Agar (BGA) (ref. 1)

Formula of CM263 from Oxoid

Proteose peptone	10.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Phenolred	0.09 g
Brilliant green	0.0047 g
Agar	12.0 g
Water	1000 ml

Preparation:
Dissolve 50g of the dehydrated medium in water by heating to the boiling point for 1 minute, adjust pH to 6.7 - 7.1 if necessary and transfer to sterile 1000 ml bottles. Do not autoclave.

Description:
Brilliant green is a selective agent. It's indicative principle is based on the ability to ferment lactose and sucrose. Phenol red is the pH indicator, which changes from yellow to red at pH 6.8 - 8.4. Therefore, lactose negative and sucrose negative bacteria like *Salmonella* grow as red-pink, white opaque colonies surrounded by brilliant red zones in the agar.
Proteus and *Pseudomonas* species may grow as small red colonies. Lactose and/or sucrose fermenting organisms are normally inhibited but may grow as yellow to greenish-yellow colonies surrounded by intense yellow-green zones in the agar. These may belong to *E. coli* or the *Klebsiella/Enterobacter* group.

Buffered peptone water (ref. 2)

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate dodecahydrate (Na2HPO4.12H2O)	9.0 g
Potassium dihydrogen phosphate (KH2PO4)	1.5 g
Water	1000 ml

Preparation:
Dissolve the peptone and chemicals in water, adjust pH to 7.0 after sterilisation. Dispense into suitable flasks and autoclave at 121°C for 20 min.

Nutrient agar (ref. 2)

Meat extract	3.0 g
Peptone	5.0 g
Agar	12 g to 18 g[1)]
Water	1000 m

[1)] Depending on the gel strength of the agar.

Preparation:
Dissolve the dehydrated medium in the water by heating if necessary. Adjust pH to ~7.0 after sterilisation, transfer into bottles and autoclave at 121°C for 20 min. Pour 15 ml of melted medium in each plate.

Rappaport-Vassiliadis Soy Peptone (RVS) Broth (ref. 3)

Base

Soy peptone	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogen phosphate (KH2PO4)	1.4 g
Dipotassium hydrogen phosphate (K2HPO4)	0.2 g
Distilled water	1000 ml

Heat to about 80°C to dissolve all ingredients. Prepare this solution the same day as the complete RVS medium is prepared.

Magnesium chloride solution

Magnesium chloride ($MgCl_2.6H2O$)	400 g	
Water		1000 ml

Dissolve the salt in the water. Because this salt is very hygroscopic, it is advisable to dissolve the entire contents of a newly opened container in distilled water. The

magnesium chloride solution can be stored unsterilised, in a dark bottle with screw cap, at room temperature for up to 2 years.

Malachite green solution

Malachite green oxalate	0.4 g
Distilled water	100 ml

Dissolve the salt in the water. The solution can be stored unsterilised, in a dark bottle with screw cap, at room temperature for up to 8 months.

Complete medium

Base	1000 ml
Magnesium chloride solution	100 ml
Malachite green solution	10 ml

Preparation:

Mix the solutions well and distribute the solution in portions of 10 ml per tube with screw cap. Autoclave at 115ºC for 15 min. Adjust the pH so that after sterilisation it is 5.2 ± 0.2 at 25ºC. Store at about 4oC for a maximum of 4 months.

Description:

This medium is used as a selective enrichment medium for the isolation of *Salmonella* from food, environment specimens and from faeces. Malachite green is the selective agent.

Salmonella species have the following characteristics when compared with other *Enterobacteriacea.*

- Ability to survive at relatively high osmotic pressure
- Multiply at relatively low pH values
- Are more resistant to malachite green and have less demanding nutritional requirements. The medium is not useful while *Salmonella* Typhi is suspected.

Brilliant green solution

Brilliant green	0.1 g
Sterile distilled water	100 ml

Iodine - Potassium iodine solution

Iodine double sublimated	16 g
Potasium iodide z.A.	20 g
Sterile distilled water	80 ml

Base (e.g. Tetrathionate Anreicherings bouillon nach Mueller Kauffman from Merck, no. 10863)

Meat extract	0.9 g
Peptone from meat	4.5 g
Yeast extract	1.8 g
Sodium chloride	4.5 g
Calcium carbonate	25.0 g
Sodium thiosulfate	40.7 g
Ox bile, dried	4.75 g
Sterile water	1000 ml
Brilliant green solution 1:1000	10 ml
Iodine-Potassium iodine solution	20 ml

Preparation:

Dissolve the tetrathionate bouillon in sterile water in a flask by shaking. Aseptically add brilliant green solution and then iodine-potassium iodine solution. Adjust pH to 7.4 - 7.8 at 25ºC. Store bouillon at about 4oC.

Description:

Tetrathionate broth is used for selective enrichment of *Salmonella*. According to ref. 2 Mueller-Kauffman Tetrathionate broth (CM343) has improved selectivity compared with Tetrathionate broth (USP) (CM671) and Tetrathionate broth (CM29) all from Oxoid, but it is inhibitory to *S*. Typhi, *S*. Pullorum, and *S*. Gallinarum (ref. 1).

Xylose lysine desoxycholate (XLD) agar (ref. 3)

Yeast extract	3.0 g
Sodium chloride	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
L-lysine hydrogen chloride	5.0 g
Sodium thiosulphate	6.8 g
Iron(III)ammonium citrate	0.8 g
Phenol red	0.08 g
Sodium desoxycholate	1.0 g
Agar	15.0 g
Distilled water	1000 ml

Preparation:

Dissolve the components in the water. Heat under constant stirring until the medium starts to boil. Avoid over-heating. Avoid preparing too large a volume of medium, as this requires prolonged heating. Immediately transfer the solution to a water bath at about 50°C, continue stirring until the medium has reached about 50°C. Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°C. Poured agar plates can be stored for a maximum of 14 days, if stored in plastic bags in the dark at about 4°C.

Add 10 ml of a 0.15% filter sterilised solution of sodium novobiocin to increase the selectivity.

Description:

Sodium desoxycholate is the selective agent and phenol red is the pH indicator. The indicative principle is based on lactose, sucrose and xylose fermentation, H2S production and lysine decarboxylation. If H_2S is produced from sodium thiosulphate, black FeS (Ferrosulfide) will develop. *Salmonella* ferments xylose, but not lactose and sucrose, decarboxylate lysine and produces H2S. *Salmonella* suspect colonies grow as red colonies with a black centre. Other bacteria that may grow on XLD agar are usually yellow and the agar will also turn yellow. Other bacteria such as *Edwardsiella* may mimic *Salmonella*.

References

1. Post D. E. (1997) Food-borne pathogens monograph number I *Salmonella*. Oxoid limited, Hampshire, England.

2. ISO 6579 :1993(E) 3rd ed. Microbiology - General guidance on methods for the detection of *Salmonella.*

3. NMKL method no. 71, 2nd ed., 1999: *Salmonella*. Detection in food. Nordic committee on food analysis.

Flow diagram for isolation/identification of *Salmonella* from Food / Animal Faceces

Non-selective pre-enrichment
25 g food / faeces in 10% phosphate buffer (36° +/-1°C, 24 h.)
↓
Selective enrichment *
0.1 ml in 10 ml Rappaport-Vassiliadis Soy Broth (41.5° +/- 0.5°C, 24 h.)
1 ml in 10 ml Tetrathionate broth (Müller-Kauffman) (36° +/-1°C, 24 h.)
↓
Isolation
XLD with an inoculation loop (36° +/-1°C, 24 h.)
BGA with an inoculation loop (36° +/-1°C, 24 h.)
↓
Suspect colonies to nutrient agar (36° +/-1°C, 24 h.)
↓
Biochemical confirmation (36° +/-1°C, 24 h.)
Triple Sugar Iron Agar (TSI) or Kliger Iron Agar (KIA)
Urea broth
Lysine Iron Agar (LIA)
Motility-Indol-Ornithine (MIO)
Citrate
↓
Serotyping
O-antigens
H-antigens
Phase I (37°C, overnight)
Phase II

* If *Salmonella* serovars Typhi or Paratyphi A are suspected: inoculate 1mL of pre-enrichment broth into 10mL of Selenite Cystine (or Selenite F) broth and incubate at 36° C (+/-1°C) for 18-24 h. Following incubation, it is advisable to inoculate the selective broth onto bismuth sulphate agar (in addition to XLD and BGA).

Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157(STEC), *Salmonella* serotypes, *Shigella* s onnei and *Shigella* flexneri

Purpose and Scope

To describe the One-Day (24-26 hour) Standardized Laboratory Protocol for Molecular Subtyping of *E. coli* O157:H7, *E. coli*Non-O157 (STEC), *Salmonella*, *Shigella sonnei* and *Shigella flexneri* by Pulsed-field Gel Electrophoresis (PFGE). To provide the PulseNet participants with the same procedure for performing PFGE of *E. coli* O157:H7, *E. coli* Non-O157(STEC), *Salmonella*, *Shigella sonnei* and *Shigella flexneri*, thus ensuring inter-laboratory comparability of the generated results.

Definitions and Terms

1. PFGE: Pulsed-field Gel Electrophoresis
2. DNA: Deoxyribonucleic acid
3. CDC: Centers for Disease Control and Prevention
4. CLRW: Clinical Laboratory Reagent Water

Biosafety Warning

Escherichia coli O157:H7, *Salmonella* serotypes, *Shigella sonnei*, and *Shigella flexneri* are human pathogens and can cause serious disease. It has been reported that less than 100 cells of *E. coli* O157:H7 may cause infection. *Shigella* species also have a low infectious dose and are demonstrated hazards to laboratory personnel. Always use Biosafety Level 2 practices (at a minimum) and extreme caution when transferring and handling strains of these genera. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plasticware and glassware that come in contact with the cultures in a safe manner.

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected with 1% Lysol/Amphyll or 90% Ethanol for at least 30 minutes if they will be washed and reused.

Day 0

Grow the culture

Streak an isolated colony from test cultures onto Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this, stab small screw cap tubes of TSA, HIA, or similar medium.with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary.

Incubate cultures at 37°C for 14-18 hours.

Day 1

Making plugs

1. Turn on shaker water bath or incubator (54-55°C), stationary water baths (55-60°C) and spectrophotometer (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).

2. Prepare TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) as follows:

2.1.10 ml of 1 M Tris, pH 8.0

2.2.2 ml of 0.5 M EDTA, pH 8.0

2.3.Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

The TE Buffer is used to make the plug agarose and also to wash lysed PFGE plugs.

3. Prepare 1% SeaKem Gold agarose in TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) for PFGE plugs as follows:

3.1.Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.

3.2.Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.

3.3.Loosen or remove cap, cover loosely with clear film, and microwave for 30 seconds; mix gently and repeat for 10 seconds intervals until agarose is completely dissolved.

3.4.Recap flask and return to 55-60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: USE HEAT-RESISTANT GLOVES WHEN HANDLING HOT FLASKS AFTER MICROWAVING.

SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

4. Label small tubes (12mm x 75mm Falcon tubes or equivalent) with culture numbers.

5. Prepare Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0) as follows:

5.1.100 ml of 1 M Tris, pH 8.0

5.2.200 ml of 0.5 M EDTA, pH 8.0

5.3.Dilute to 1000 ml with sterile Ultrapure water (CLRW)

6. Transfer 2 ml of Cell Suspension Buffer (CSB) to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, orcolorimeter. Keep suspensions on ice if you have more than 6 cultures to process or refrigerate cell suspensions if you cannot adjust their concentration immediately.

7. Adust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.

7.1.Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 1.00 (range of 0.8-1.0)

7.2.Dade Microscan Turbidity Meter:

7.2.1.0.40-0.45 (measured in Falcon 2054 tubes)

7.2.2.0.58-0.63 (measured in Falcon 2057 tubes)

7.3.bioMérieux Vitek colorimeter: 17-18% transmittance (measured in Falcon 2054 tubes)

The values in step 7 give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

Casting Plugs

1. Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10-15seconds and mix; repeat for 5-10 seconds intervals until agarose is completely melted. This agarose melts rapidly!

Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot 300-500 µl into small tubes and store at -20°C until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

2. Transfer 400 µl adjusted cell suspensions to labeled 1.5-ml microcentrifuge tubes.

3. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl are needed for 10 cell suspensions.)

4. Add 400 µl melted 1% SeaKem Gold agarose to 400 µl cell suspension; mix by gently pipetting mixture up and down a few times. Over-pipetting can cause DNA shearing. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60°C).

5. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is required. Allow plugs to solidify at room temperature for 10-15 minutes. They can also be placed in the refrigerator (4°C) for 5 minutes

If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of around 10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

Lysis of Cells in Agarose Plugs

Two plugs (reusable molds) or 3 – 4 plugs (disposable molds) of the same strain can be lysed in the same 50ml tube.

1 .Label 50ml polypropylene screw-cap or 50ml Oak Ridge tubes with culture numbers.

2. Prepare Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) as follows:

2.1.50 ml of 1 M Tris, pH 8.0

2.2.100 ml of 0.5 M EDTA, pH 8.0

2.3.100 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)

2.4.Dilute to 1000 ml with sterile Ultrapure water (CLRW)

3. Calculate the total volume of Cell Lysis/Proteinase K Buffer needed as follows:

3.1.5 ml Cell Lysis Buffer is needed per tube

e.g., 5 ml x 10 tubes = 50 ml

3.2.25 µl Proteinase K stock solution (20 mg/ml) is needed per tube of the cell lysis buffer

e.g., 25 µl x 10 tubes = 250 µl

4. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

The final concentration of Proteinase K in the lysis buffer is 0.1 mg/ml, and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

5. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

6. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6-mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. Be sure plugs are under buffer and not on side of tube.

The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

7. Remove tape from reusable mold. Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll or other suitable disinfectant. Soak them for 15 minutes before washing them. Discard disposable plug molds or disinfect them in 90% ethanol for 30-60 minutes if they will be washed and reused.

8. Place tubes in rack and incubate in a 54-55°C shaker water bath or incubator for 1.5-2 hours with constant and vigorous agitation (150-175 rpm). If lysing in water bath, be sure water level is above level of lysis buffer in tubes.

9. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55°C so that plugs can be washed two times with 10-15 ml water(200-300 ml for 10 tubes)

Washing of Agarose Plugs After Cell Lysis

Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55°C.However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50°C for the following washing steps.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55°C to each tube and shake the tubes in a 54-55°C water bath or incubator for 10-15 minutes.

3.Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.

4. Pre-heat enough sterile TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55°C water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.

5. Pour off water, add 10-15 ml pre-heated (54-55°C) sterile TE Buffer, and shake the tubes in 54-55°C water bath or incubator for 10-15 minutes.

6. Pour off TE and repeat wash step with pre-heated TE three more times.

7. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4°C until needed. Plugs can be transferred to smaller tubes for long term storage.

If restriction digestion is to be done the same day, complete Steps 1-3 of next section (Restriction Digestion) during last TE wash step for optimal use of time.

Restriction Digestion of DNA in Agarose Plugs

A small slice of the plug should be digested with the primary restriction enzyme because less enzyme is required and other slices of the plug can be subjected to restriction analysis with secondary or tertiary enzymes, according to the table below. E. coli species, Salmonella, and Shigella sonnei utilize XbaI as the primary enzyme and BlnI as the secondary enzyme.

Shigella flexneri are tested with NotI as the primary enzyme and XbaI as the secondary enzyme. The use of a secondary (or tertiary) enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

Organism	Primary Enzyme	Secondary Enzyme	Tertiary Enzyme
***E. coli* O157**	*Xba*I (50U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30U/sample)
***E. coli* non-O157**	*Xba*I (50U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30U/sample)
Salmonella	*Xba*I (50U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30U/sample)
S. sonnei	*Xba*I (50U/sample)	*Bln*I/*Avr*II (30U/sample)	*Spe*I (30U/sample)
S. flexneri	*Not*I (50U/sample)	*Xba*I (50U/sample)	*Spe*I (30U/sample)

1. Label 1.5-ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for Salmonella ser. Braenderup H9812ı standards.

2. Pre-Restriction Incubation Step: Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

Reagent	µl/Plug Slice	µl/10Plug Slices	µl/15 Plug Slices
CLRW	180 µl	1800 µl	2700 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

3. Add 200 µl diluted restriction buffer (1X) to labeled 1.5-ml microcentrifuge tubes.

4. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.

5. Cut a 2.0 to 2.5mm wide slice from each test samples and the appropriate number of S. ser. Braenderup H9812 standards with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted restrictionbuffer. Be sure plug slice is under buffer. Replace rest of plug into the original tube that contains 5 ml TE buffer and store at 4°C.

PulseNet recommends that the combs with larger teeth (10 mm wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

5.1.Incubate sample and control plug slices in a 37°C water bath for 5-10 minutes or at room temp for 10-15 minutes.

5.2.After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipet tip and that plug slice is not discarded with pipet tip.

6. Prepare the restriction enzyme master mix according to the following table. May mix in the same tube that was used for the diluted restriction buffer.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
CLRW	173 µl	1730 µl	2595 µl
10X Restriction Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
XbaI (10U/ µl)	5 µl	50 µl	75 µl
Total Volume	200 µl	2000 µl	3000 µl

Keep vial of restriction enzyme on ice or in insulated storage box (-20°C) at all times.

Addition of Bovine Serum Albumin (BSA; highly recommended): Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

7. Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; be sure plug slices are under enzyme mixture.

8. Incubate sample and control plug slices in 37˚C water bath for 1.5-2 hours.

9. If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (Casting an Agarose Gel) approximately 1 hour before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

Casting an Agarose Gel

1. Confirm that water bath is equilibrated to 55-60°C.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

Reagent	Volume (ml)	Volume(ml)
5X TBE Stock	200	220
CLRW	1800	1980
Total Volume	2000	2200

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:

3.1. Weigh appropriate amount of SKG into 500 ml screw-cap flask.

Mix 1.0 g agarose with 100 ml 0.5X TBE for 14cm-wide gel form (10 wells)

Mix 1.5 g agarose with 150 ml 0.5X TBE for 21cm-wide gel form (15 wells)

3.2. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.

4. Loosen cap and microwave for 60 seconds; mix gently and repeat for 15 second intervals until agarose is completely dissolved.

5. Return flask to 55-60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

SAFETY WARNING: USE HEAT-RESISTANT GLOVES WHEN HANDLING HOT FLASKS AFTER MICROWAVING.

Agarose LFTM (Amresco, X174) and Certified Megabase Agarose (Bio-Rad, 161-3108) are the only acceptable alternatives to SeaKem Gold, at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

6. A small volume (2-5 ml) of melted and cooled (55-60°C) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

7. Remove restricted plug slices from 37°C water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

8. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:

8.1.Load S. ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10 well gel) or on teeth 1, 5, 10, 15 (15 well gel).

8.2.Load samples on remaining teeth and note locations.

9. Remove excess buffer with a kimwipe. Allow plug slices to air dry on the comb for 3-5 minutes or seal them to the comb with 1% SKG agarose (55-60°C).

10. Position comb in gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform.

11. Carefully pour the agarose (cooled to 55-60°C) into the gel form and remove any bubbles or debris.

12. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run).

13.Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of about 70) and cooling module (14°C) approximately 30 minutes before gel is to be run.

14. Remove comb after gel solidifies for 30-45 minutes.

15. Fill in wells of gel with melted and cooled (55-60°C) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

Loading Restricted Plug Slices into the Wells (optional alternate method)

1. Follow steps 1-4 in the section Loading Restricted Plug Slices on the Comb.

Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that front part (side with small metal screws) and teeth face the bottom of gel and the bottom edge of the comb is 2 mm above the surface of the gel platform.

2. Cool melted SKG agarose in 55-60°C water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)

4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting at about 70), and cooling module (14°C) approximately 30 minutes before gel is to be run.

5. Remove restricted plug slices from 37°C water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies for at least 30 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.

7.1.Load *S.* ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10-well gel) or in wells 1, 5, 10, 15 (15 well gel).

7.2.Load samples in remaining wells.

Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55-60°C). Allow to harden for 3-5 minutes. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

Electrophoresis Conditions

***Escherichia coli* O157:H7 and *Shigella sonnei* strains restricted with *Xba*I or *Avr*II (*Bln*I):**

- Select following conditions on CHEF Mappero Auto Algorithm
  - o 30 kb: low MW o600 kb: high MW
  - o Select default values except where noted by pressing "enter". oChange run time to 18-19 hours (See note below)
  - o (Default values: Initial switch time = 2.16 s; Final switch time = 54.17 s)
- Select following conditions on CHEF-DR III
  - oInitial switch time: 2.2 s
  - oFinal switch time: 54.2 s
  - oVoltage: 6 V
  - oIncluded Angle: 120°
  - oRun time: 18-19 hours (See note below)
- Select following conditions on CHEF-DR II
  - oInitial A time: 2.2 s
  - o Final A time: 54.2 s
  - o Start ratio: 1.0 (if applicable) oVoltage: 200 V
  - o Run time: 19-20 hours (See note below)

***Salmonella* strains restricted with *Xba*I or *Avr*II (*Bln*I):**

- Select following conditions on CHEF Mappero Auto Algorithm
  - o 30 kb: low MW o700 kb: high MW
  - o Select default values except where noted by pressing "Enter." oChange run time to 18-19 hours (See note below)
  - o (Default values: Initial switch time = 2.16 s; Final switch time = 63.8 s)
- Select following conditions on CHEF DR-III
  - oInitial switch time: 2.2 s
  - oFinal switch time: 63.8 s
  - oVoltage: 6 V
  - oIncluded Angle: 120°
  - oRun time: 18-19 hours (See note below)
- Select following conditions on CHEF DR-II.
  - oInitial A time: 2.2s
  - o Final A time: 63.8 s
  - o Start Ratio: 1.0 (if applicable) oVoltage: 200 V
  - o Run time: 19-20 hours (See note below) o

Non-O157 *Escherichia coli* strains restricted with *Xba*I or *Avr*II (*Bln*I):

- Select following conditions on CHEF Mappero Auto Algorithm
  - o 50 kb: low MW o400 kb: high MW
  - o Select default values except where noted by pressing "Enter." oChange run time to 18-19 hours (See note below)
  - o (Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s)
- Select following conditions on CHEF DR-III
  - oInitial switch time: 6.76 s
  - oFinal switch time: 35.38 s
  - oVoltage: 6 V
  - oIncluded Angle: 120°
  - oRun time: 18-19 hours (See note below)
- Select following conditions on CHEF DR-II.
  - oInitial A time: 6.76 s
  - o Final A time: 35.38 s
  - o Start Ratio: 1.0 (if applicable) oVoltage: 200 V
  - o Run time: 19-20 hours(See note below)

Shigella flexneri* strains restricted with *Not*I or *XBAI:

- Select following conditions on CHEF Mappero Auto Algorithm
  - o 50 kb: low MW o400 kb: high MW
  - o Select default values except where noted by pressing "Enter." oChange the switch times to the following values:
  - o Initial switch time: 5 seconds oFinal switch time: 35 seconds
  - o Change run time to 18-19 hours (See note below)
  - o (Default values: Initial switch time = 6.76 s; Final switch time = 35.38 s)
- Select following conditions on CHEF DR-III
  - oInitial switch time: 5 s
  - oFinal switch time: 35 s
  - oVoltage: 6 V
  - oIncluded Angle: 120°
  - oRun time: 18-19 hours(See note below)
- Select following conditions on CHEF DR-II.
  - oInitial A time: 5 s
  - o Final A time: 35 s
  - o Start Ratio: 1.0 (if applicable) oVoltage: 200 V
  - o Run time: 19-20 hours(See note below)

The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the S. ser. Braenderup H9812 standard migrates within 1.0-1.5 cm of the bottom of the gel.

Make note of the initial milliamp (mA) reading on the instrument. The initial mA should be between 110-150 mA. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

Day 2

Staining and Documentation of an Agarose Gel

The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the Important PulseNet Documents forum on CDC Team for additional information.

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide. Dilute 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of Ultrapure water (CLRW) (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20-30minutes in covered container.

Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the Material Safety Data Sheets (MSDS) provided by the vendor or manufacturer.

Currently, the only acceptable alternative stain options are GelRedTM (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. Importantly, if one of the alternative stains is used, the detaining steps should be omitted.

2. Destain gel in approximately 500 ml reagent grade water for 60-90 minutes; change water every 20 minutes. Capture image a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system. If too much background is observed de- stain for an additional 30-60 minutes.

3. Follow directions given with the imaging equipment to save gel image as a *.1sc or *.scn file; convert this file to *.tif file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands. Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L Ultrapure water (CLRW) or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 minutes before draining water from chamber and hoses.

5. If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

Please note the following if PFGE results do not have to be available within 24-28 hours:

- *Plugs can be lysed for longer periods of time (3-16 hours).*
- *The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time(30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.*

USE OF TRADE NAMES AND COMMERCIAL SOURCES IS FOR IDENTIFICATION PURPOSES ONLY AND DOES NOT IMPLY ENDORSEMENT BY CDC OR THE U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES.

CLIA Laboratory Procedure Manual Requirements

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE

Tris: EDTA Buffer, pH 8.0: (TE, 10 mM Tris: 1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0

2 ml of 0.5 M EDTA, pH 8.0

Dilute to 1000 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer: (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)

50 ml of 1 M Tris, pH 8.0

100 ml of 0.5 M EDTA, pH 8.0

100 ml 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl) OR 5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)[2]

Dilute to 1000 ml with Sterile Ultrapure water (CLRW)

Add 25 µl Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer just before use for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

Restriction Enzyme Master Mix: *Avr*II, *Bln*I, *Spe*I (30U/plug slice)

Reagent	µl/Plug Slice	µl/Plug Slices	µl/15Plug Slices
CLRW	175 µl	1750 µl	2625 µl
H Buffer	20 µl	200 µl	300 µl
BSA (10mg/ml)	2 µl	20 µl	30 µl
Enzyme (10U/µl)	3 µl	30 µl	45 µl
Total Volume	200 µl	2000 µl	3000 µl

Keep vials of restriction enzyme on ice or in an insulated storage box (-20°C) at all times.[2] If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50- 60ºC for 30-60 minutes, or leave at room temperature for about 2 hours to completely dissolve the Sarcosyl

CURRICULUM VITAE

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Education

1999-2004	High school, Srisawatwittayakarn School, Nan, Thailand
2005-2010	Bachelor's degree, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand

Scholarship

2012-2014	National Science and Technology Development Agency (NSTDA)

Work experience

2010-2012	Technical Advisor, Novartis Thailand Co. Ltd., Bangkok, Thailand
2012	Clinician, Sai Mun Veterinary Clinic, Chiang Mai, Thailand
2013-now	Clinician, Pasusad Wetchakan Clinic, Chiang Mai, Thailand
2013-now	Clinician and owner, Community Vet Clinic, Chiang Mai, Thailand

Present specialty Statistical computer-program (R®, Stata™, Epiinfo®), Electric guitars, Drums, Basketball

Workshop "Joint workshop on scientific writing in field epidemiology,2014" at Champasak Grand Hotel, Champasak Province, Lao PDR. 25th Feb-1st Mar,2014

Field practice

2009	Pharmatech co. ltd., Nakon pathom, Thailand
2008	Small animal hospital, Chiang Mai University, Chiang Mai, Thiland
2008	Chiang Mai night safari zoo, Chiang Mai, Thailand
2007	Chiang Mai zoo, Chaing Mai, Thailand
2006	Donkaew swine farms, Chiang Mai, Thailand