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 25^{o}C. 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 50ºC, continue stirring until the medium has reached about 50ºC. Adjust the pH so that after heating it is 7.4 ± 0.2 at 25ºC. Poured agar plates can be stored for a maximum of 14 days, if stored in plastic bags in the dark at about 4ºC.

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).

Extraction Protocol: Chelex

This is a fast, cheap, and effective method of DNA extraction. Because this is the first step towards PCR and amplifying your template DNA, you must maintain excellent sterile technique to prevent the contamination of your DNA extractions. Always use a negative chelex control (below) to evaluate your technique during the extraction phase.

Work in LOW DNA part of lab.

1. Remove premade tubes filled with 300uL 10% Chelex from refridgerator. You will need for each sample, plus 1extra for a control. Handle the container with gloves and shake out the number you need into your gloved hand. Do not put extra tubes back into jar.
2. Label each Chelex tube to correspond to your sample listed on your Chelex worksheet. Label the tubes on the cap. Put as much information as you can manage, but keep labels legible. Adding initials and a date is always a good idea. Avoid labeling on the side of tube as this writing can be washed off during the incubation stage.
3. Turn on heating block. Set to 95°C. Fill holes with ddH2O water.
4. Dip forceps into ethanol, then wave forceps through flame of an alcohol burner to ignite. When you are certain that the flame on the forceps has extinguished, repeat 2 times.
5. Using the sterile forceps, remove a small piece of tissue from your sample, uncap the tube of chelex, place sample in the appropriately labeled tube and close lid. The piece of tissue should be big enough to be visible, but not so big as to be easily visible. Imagine cutting a 0.2mm section of a standard staple. This is plenty big. Too much tissue may inhibit your reactions. The piece of tissue should be about as big as a period.
6. Repeat (step 5) with each sample in a new Chelex tube, being sure to sterilize forceps 3 times between samples (step 4). When finished, make a negative Chelex control by dipping your sterilized forceps into a tube of Chelex slurry. (It may be necessary to wipe excess tissue from forceps with a kimwipe prior to flame sterilization)

7. When finished with all tubes, vortex samples in chelex slurry for 10-15 seconds. Be sure lids are snapped on tightly before begining
8. Spin samples briefly (10-15 sec) at high speed in a microcentrifuge. This step is to ensure that the sample is inside the slurry of Chelex.
9. Incubate samples for 20 minutes at 95°C. The block temperature may drop slightly when doing this step. This drop is normal. Check tubes while incubating to ensure that lids have not popped off.
10. Vortex samples again for 10-15 seconds (Be careful asmsteam may pop lid off of centrifuge tube. Hold lids down).
11. Spin tubes again at high speed in microcentrifuge to ensure that all contents are in the bottom of the microcentrifuge tube.
12. Samples are ready to use (or not, see below).

ONLY

USE SUPERNATE FOR PCR REACTIONS.

CHELEX BEAD WILL INACTIVATE TAQ!

Notes:

Chelex is notorious for being as fickle as it is cheap and easy. Here are some tips for good amplifications:

1. Sometimes samples work best if used immediately. Sometimes it is better to wait overnight before using them. Experiment and find what works for your species. Results can vary by taxa and by individuals.
2. When doing initial PCRs, do a serial dilution of template. The amount of a Chelex DNA extraction used in a PCR can be as high as half of the volume of the PCR or as low as 1 microliter of a 1:10,000 dilution. I find that 1 microliter of a 1:1 is good for most applications, but if your PCR doesn't work initially, vary template concentration.
3. If you don't get amplifications from your PCR the first time with a Chelex extract, repeat the vortex, spin, incubate, vortex, spin, sit overnight procedure described above. Often this will make a negative PCR work.

QIAGEN QIAmp mini Kit

1. Inoculate 3-5ml PY media with the desired strain, and incubate at 37°C with shaking overnight.
2. Harvest 2 ml of culture by centrifugation (7500rpm, 10min, room temperature (RT)).
3. Re-suspend pellet with 180µl buffer ATL and incubate for 30 mins at 37°C.

   [Proceed from section 3, page 34 QIAGEN manual]

4. Add 20µl Proteinase K (in kit) to the suspension and mix by vortexing.
5. Incubate at 56°C for 30-60 min.
6. Spin down to remove condensation from caps and add 4 µl RNase (100mg/ml) and 200µl Buffer AL (in kit).
7. Incubate at 70°C for 10 min.
8. Add 200µl of 95-100 % (v/v) ethanol to the suspension and vortex.
9. Pipette the suspension into a QIAmp column in a 2 ml collection tube, and centrifuge (8000 rpm, 1min, RT).
10. Discard the flow through and place the column in a clean 2 ml collection tube.
11. Add 500 µl Buffer AW1 (in kit) and centrifuge (8000 rpm, 1 min, RT).
12. Discard the flow-through and add 500µl of Buffer AW2 (in kit), and centrifuge for 3 min at 14,000 rpm. (RT) to dry the QIAmp column membrane.
13. Place the QIAmp column in a clean micro-centrifuge tube and pipette 50µl Buffer AE (in kit) onto the column membrane.
14. Leave the column for 1 min at RT, before centrifugation at 8000 rpm for 1 min to elute the genomic DNA, and store at 4°C or long term at -20°C

Protocols used for MLST of *Salmonella enterica*

Genes

The *S.* enterica MLST scheme uses internal fragments of the following seven house-keeping genes:

thrA (aspartokinase+homoserine dehydrogenase)
purE (phosphoribosylaminoimidazole carboxylas)
sucA (alpha ketoglutarate dehydrogenase)
hisD (histidinol dehydrogenase)
aroC (chorismate synthase)
hemD (uroporphyrinogen III cosynthase)
dnaN (DNA polymerase III beta subunit)

PCR Amplification

The primer pairs we use for the PCR amplification of internal fragments of these genes are:

	Product length:
thrA: F 5'-GTCACGGTGATCGATCCGGT-3' **recommended**	852 bp
thrA: R 5'-CACGATATTGATATTAGCCCG-3' **recommended**	
thrA: R1 5'-GTGCGCATACCGTCGCCGAC-3' (also Seq)	
purE: F 5'-ATGTCTTCCCGCAATAATCC-3'	510 bp
purE: F1 5'-GACACCTCAAAAGCAGCGT'-3' **recommended**	
purE: R 5'-TCATAGCGTCCCCCGCGGATC-3'	
purE: R1 5'-CGAGAACGCAAACTTGCTTC-3'	
purE: R2 5'-AGACGGCGATACCCAGCGG-	

3' **recommended**

sucA: F 5'-AGCACCGAAGAGAAACGCTG-3' 643 bp

sucA: F1 5'-CGCGCTCAAACAGACCTAC-

3' **recommended**

sucA: R 5'-GGTTGTTGATAACGATACGTAC-3'

sucA: R1 5'-GACGTGGAAAATCGGCGCC-

3' **recommended**

hisD: F 5'-GAAACGTTCCATTCCGCGCAGAC-3' 894 bp

hisD: F1 5'-GAAACGTTCCATTCCGCGC-

3' **recommended**

hisD: R 5'-CTGAACGGTCATCCGTTTCTG-3'

hisD: R1 5-GCGGATTCCGGCGACCAG-3' **recommended**

aroC: F 5'-CCTGGCACCTCGCGCTATAC- 826 bp

3' **recommended**

aroC: R 5'-CCACACACGGATCGTGGCG-

3' **recommended**

hemD: F 5'-ATGAGTATTCTGATCACCCG-3' 666 bp

hemD: F1 5'-GAAGCGTTAGTGAGCCGTCTGCG-

3' **recommended**

hemD: R 5'-ATCAGCGACCTTAATATCTTGCCA-

3' **recommended**

dnaN: F 5'-ATGAAATTTACCGTTGAACGTGA-3' 833 bp

dnaN: R 5'-AATTTCTCATTCGAGAGGATTGC-

3' **recommended**

dnaN: R1 5'-CCGCGGAATTTCTCATTCGAG-

3' **recommended** (also Seq)

An annealing temperature of 55° C is fine for all genes.

Sequencing

Together with the recommended PCR primers above, we recommend using the following sequencing primers at 50C

aroC: aroC_sF1(GGCGTGACGACCGGCAC) and aroC_sR1 (AGCGCCATATGCGCCAC)
dnaN: dnaN_sF (CCGATTCTCGGTAACCTGCT) and dnaN_sR1 (ACGCGACGGTAATCCGGG)
hemD: hemD_sF2 (GCCTGGAGTTTTCCACTG) and hemd_sR (GACCAATAGCCGACAGCGTAG)
hisD: hisD_sF (GTCGGTCTGTATATTCCCGG) and hisD_sR (GGTAATCGCATCCACCAAATC)
purE: purE_sF1 (ACAGGAGTTTTAAGACGCATG) and purE_sR1 (GCAAACTTGCTTCATAGCG)
sucA: sucA_sF1 (CCGAAGAGAAACGCTGGATC) and sucA_sR (GGTTGTTGATAACGATACGTAC)
thrA: thrA_sF (ATCCCGGCCGATCACATGAT) and thrA_sR1 (ACCGCCAGCGGCTCCAGCA)

Our previous recommendations were to use the PCR primers for *sucA*, *thrA*R1 and *dnaN*R1 for sequencing as well as the other primers listed below:

thrA: sF 5'-ATCCCGGCCGATCACATGAT-3'
thrA: sR 5'-CTCCAGCAGCCCCTCTTTCAG-3'

purE: sF 5'-CGCATTATTCCGGCGCGTGT-3'
purE: sF1 5'-CGCAATAATCCGGCGCGTGT-3'
purE: sR 5'-CGCGGATCGGGATTTTCCAG-3'
purE: sR1 5'-GAACGCAAACTTGCTTCAT-3'

sucA: sF 5'-AGCACCGAAGAGAAACGCTG-3'
sucA: sR 5'-GGTTGTTGATAACGATACGTAC-3'

hisD: sF 5'-GTCGGTCTGTATATTCCCGG-3'
hisD: sR 5'-GGTAATCGCATCCACCAAATC-3'

aroC: sF 5'-GGCACCAGTATTGGCCTGCT-3'

aroC: sR 5'-CATATGCGCCACAATGTGTTG-3'

hemD: sF 5'-GTGGCCTGGAGTTTTCCACT-3'

hemD: sF1 5'-ATTCTGATCACCCGCCCCTC-3'

hemD: sR 5'-GACCAATAGCCGACAGCGTAG-3'

dnaN: sF 5'-CCGATTCTCGGTAACCTGCT-3'

dnaN: sR 5'-CCATCCACCAGCTTCGAGGT-3'

NexteraXTDNALibraryPrep Protocol Guide

For Research Use Only. Not for use in diagnostic procedures.

Tagment Genomic DNA

Amplify Libraries

Clean Up Libraries

Check Libraries

Normalize Libraries

Pool Libraries

Tagment Genomic DNA

Preparation 1 Save the following tagmentation program on the thermal cycle Choose the preheat lid option

55°Cfor 5 minutes

Hold at 10°C

Procedure

1. Add the following items in the order listed to a new Hard-Shell skirted PCR plate. Pipette to mix.

Item	Volume (µl)
TD	10
Normalized gDNA	5

2. Add 5 µl ATM. Pipette to mix.
3. Centrifuge at 280 × g at 20°Cfor 1 minute.
4. Place on the thermal cycler and run the tagmentation program.
5. Add 5 µl NT. Pipette to mix.
6. Centrifuge at 280 × g at 20°Cfor 1 minute.
7. Incubate at room temperature for 5 minutes.
8. [Optional] To assess tagmentation, run 1 µl sample on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip.

AmplifyLibraries

Preparation

1. Save the following program on the thermal cycler: -Choose the preheat lid option.

-72°C for 3 minutes

-95°C for 30 seconds

-12 cycles of:

-95°C for 10 seconds

-55°C for 30 seconds

-72°C for 30 seconds

-72°C for 5 minutes

-Hold at 10°C

Procedure

1. [24 libraries] Arrange the index primers in the TruSeq Index Plate Fixture as follows. -Arrange Index 1 (i7) adapters in columns 1–6 of the TruSeq Index Plate Fixture. -Arrange Index 2 (i5) adapter in rows A–D of the TruSeq Index Plate Fixture.
2. [96 libraries] Arrange the index primers in the TruSeq Index Plate Fixture as follows. -Arrange Index 1 (i7) adapters in columns 1–12 of the TruSeq Index Plate Fixture. -Arrange Index 2 (i5) adapter in rows A–H of the TruSeq Index Plate Fixture.
3. Using a multichannel pipette, add 5 µl of each Index 1 (i7) adapterdown each column. Replace the cap on each i7 adapter tube with a new orange cap.
4. Using a multichannel pipette, add 5 µl of each Index 2 (i5) adapter across each row. Replace the cap on each i5 adapter tube with a new white cap.
5. Add 15 µl NPM. Pipette to mix.
6. Centrifuge at 280 × g at 20°C for 1 minute.
7. Place on the thermal cycler and run the PCR program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up Libraries

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
RSB	-25°C to -15°C	Thaw at room temperature. RSB can be stored at 2°C to 8°C after the initial thaw.
AMPure XP Beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2. Prepare fresh 80% ethanol from absolute ethanol.
3. Centrifuge at 280 × g at 20°C for 1 minute.
4. Transfer 50 µl PCR product from the PCR plate to a new midi plate. Add 30 µl AMPure XP beads.
5. Shake at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 5 minutes.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes). Remove and discard all supernatant.
8. Wash 2 times with 200 µl 80% EtOH.
9. Using a 20 µl pipette, remove residual 80% EtOH. Air-dry on the magnetic stand for 15 minutes. Remove from the magnetic stand.
10. Add 52.5 µl RSB.
11. Shake at 1800 rpm for 2 minutes.
12. Incubate at room temperature for 2 minutes.
13. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
14. Transfer 50 µl supernatantto a new TCY plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

CheckLibraries

1. [Optional] Run 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip.

NormalizeLibraries

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
LNA1	-25°C to -15°C	Prepare under a fume hood. Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNB1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNW1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNS1	Room temperature	Bring to room temperature.

Procedure

1. Transfer 20 µl supernatant to a new midi plate.
2. [96 samples] Add 4.4 ml LNA1 to a new 15 ml conical tube.
3. Thoroughly resuspend LNB1. Pipette to mix.
4. Transfer 800 µl LNB1 to the 15 ml conical tube. Invert to mix.
5. Pour the bead mixture into a trough and add 45 µl combined LNA1/LNB1.
6. Shake at 1800 rpm for 30 minutes.

7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Remove and discard all supernatant.
9. Wash 2 times with 45 µl LNW1. Add 30 µl 0.1 N NaOH.
10. Shake at 1800 rpm for 5 minutes.
11. During the 5 minute elution, label a new 96-well PCR plate SGP.
12. Add 30 µl LNS1 to the SGP plate. Set aside.
13. After the 5 minute elution, make sure that all samples are resuspended. Pipette to mix.
14. Shake at 1800 rpm for 5 minutes.
15. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
16. Transfer the supernatant from the midi plate to the SGP plate.
17. Centrifuge at 1000 × g for 1 minute.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Pool Libraries

Preparation

1. To prepare for the sequencing run, begin thawing reagents according to the instructions for your sequencing instrument.
2. If the SGP plate was stored frozen at -25°C to -15°C, thaw at room temperature. Pipette to mix.

Procedure

1. Centrifuge at 1000 × g at 20°C for 1 minute.
2. Transfer 5 µl from the SGP plate to a new PCR 8-tube strip.
3. Label a new Eppendorf tube PAL.
4. Transfer the contents of the PCR 8-tube strip to the PAL tube. Invert to mix.
5. Dilute pooled libraries to the loading concentration for the sequencing instrument you are using. See the denature and dilute libraries guide for your instrument.
6. Store unused pooled libraries in the PAL tube and SGP plate at -25°C to -15°C for up to 7 days.

CURRICULUM VITAE

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Education

2007-2012	Faculty of Veterinary Medicine, Chiang Mai University
1994-2006	Dara Academy school, Chiang Mai
1992-1993	Sradetasin school, Chiang Mai

Work experience

2015-2016	Academic staff of Sheppard lab, Medical school, Swansea, UK
2013-now	Clinician and owner, Community Vet Clinic, Chiang Mai, Thailand
2013-2014	Clinician, Pasusad Wetchakan Clinic, Chiang Mai, Thailand
2012-2013	Chiang Mai – Lamphun Clinic, Chiang Mai, Thailand
2012	Clinician, San-sai hospital, Chiang Mai, Thailand
2012	Clinician, Ban-Mor-Ruk-Sad hospital, Chiang Mai, Thailand

Present specialty	Molecular epidemiology, Antimicrobial resistance, Microbiology, Violin.
Scholarship	2012-2017, NSTDA University Industry Research Collaboration, NUI-RC
Workshop	- The scientific conference and opening of the MRC CLIMB centre at Swansea University, UK. 23rd October, 2015

- "Sakura Science Plan Project" in topic Diagnostic Methods for infectious disease of animal at Azabu university, Japan, 7th-16th December, 2014
- "Joint workshop on scientific writing in field epidemiology,2014" at Champasak Grand Hotel, Champasak Province, Lao PDR. 25th Feb-1st Mar,2014
- Participant of training Real time PCR technique for gene quantification and detection 19th -20th Feb 2014 at Gibthai Training Center , Chiang Mai, Thailand

Field practice

2012	Small animal teaching hospital, Michigan State, USA
2011	Fhasai animal Hospital, Chiang Mai, Thailand
2010	Small animal teaching hospital, Chiang Mai, Thailand
	Nuengrynthai Sadtawaphaet Clinic, Chiang Mai, Thailand
2009	Chiang Mai Zoo, Chiang Mai, Thailand
	Chiang Mai Zoo Aquarium, Chiang Mai, Thailand
2008	Yupa Pig Farm, Chiang Mai, Thailand
	Chiang Mai, Livestock Research and Breeding center, Chiang Mai, Thailand

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