

1) **Laboratory procedures recommended for the conventional polymerase chain reaction test for *Salmonella* Enteritidis.**

(a) Sample Enrichment:

Samples (drag swabs, chick paper swabs, etc.) are enriched in Tetrathionate enrichment for 18-24 hours 37 or 42.0°C (see § 147.12) and sub cultured onto Modified Semi-solid Rappaport-Vassiliadis (MSRV) Enrichment (see §147.12).

(b) Quality Control:

A positive control, known *Salmonella* Enteritidis or ATCC strain, from BHI broth should be inoculated onto MSRV media, incubated for 18-24 hours and 1-3 plugs harvested from the zone of white precipitate growth outside the initial inoculation site. A negative control is harvested plugs from an area of uninoculated MSRV plate.

(c) DNA extraction:

DNA is extracted from 1-3 plugs (~100 µL) of MSRV agar in the zone of migration outside of initial inoculation site by boiling in 100µl PCR grade water for 10 minutes or by another DNA extraction method. Samples are cooled to room temperature before PCR use or stored at 2-8°C, if PCR is not performed immediately. For the boiling method, the extracted samples are spun at 16,000 rcf for 3 minutes. The DNA is contained in the supernatant.

(d) Primer Selection:

The SE specific primers are:

sdf I (forward) – TGTGTTTTATCTGATGCAAGAGG

sdf I (reverse) – CGTTCTTCTGGTACTTACGATGAC.

The internal control primers are:

rpl I (forward) – GGGTGATCAGGTTAACGTTAAAG

rpl I (reverse) – CTTCGTGTTCCGCCAGTGGTACGC.

(e) Polymerase chain reaction:

The following multiplex-PCR reaction (2 sets of primers per reaction tube) should be set up in a 200µl PCR tube or a 25 µl PCR tube, in a clean environment.

Reaction Mix	50 µL Volume	25 µL Volume	Final Concentration	
10X PCR Gold Buffer	5µl	2.5µl	1X	
MgCl ₂ (25mM)		5µl	2.5µl	2.5mM
10mM dNTP mix		3µl	1.5µl	150µM each
<i>sdf I</i> Forward Primer	4µl	2.0µl	0.4µM	
<i>sdf I</i> Reverse Primer	4µl	2.0µl	0.4µM	
<i>rpl I</i> Forward Primer	4µl	2.0µl	0.4µM	
<i>rpl I</i> Reverse Primer	4µl	2.0µl	0.4µM	
Amplitaq Gold Polymerase	0.5µl	0.25µl	2.5U	
Sterile PCR Grade Water		15.5µl	5.25µl	

2) **Laboratory procedures recommended for the real-time polymerase chain reaction test for *Salmonella sp. Group D***

(a) Sample Enrichment:

Samples (drag swabs, chick paper swabs, etc.) are enriched in Tetrathionate enrichment for 24 hours 37 or 42° C (see § 147.12) and subcultured onto Modified Semi-solid Rappaport-Vassiliadis (MSRV) Enrichment (see §147.12).

(b) Quality Control:

A positive control, known *Salmonella* Enteritidis or ATCC strain, from BHI broth should be inoculated onto MSRV media, incubated for 18-24 hours and 1-3 plugs harvested from the zone of white precipitate growth outside the initial inoculation site. A negative control is harvested plugs from an area of uninoculated MSRV plate.

(c) DNA extraction:

DNA is extracted from 1-3 plugs (~100 µL) of MSRV agar in the zone of migration outside of initial inoculation site by boiling in 100µl PCR grade water for 10 minutes or by another DNA extraction method. Samples are cooled to room temperature before PCR use or stored at 2-8°C, if PCR is not performed immediately. For the boiling method, the extracted samples are spun at 16,000 rcf for 3 minutes. The DNA is contained in the supernatant.

(d) Primer & Probe Selection:

sefAF (forward): 22 bp sequence 5'GGCTTCGGTATCTGGTGGTGTA3' (50 nM final concentration); (example: use volumes below if stock primer is 10 µM concentration).

sefAR (reverse): 24 bp sequence 5'GGTCATTAATATTGGCCCTGAATA3' (900 nM concentration); (example: use volumes below if stock primer is 10 µM concentration).

sefAPR (probe): 25 bp sequence 5'FAM/CCACTGTCCCGTTCGTTGATGGACA/TAMRA' or comparable quencher (250 nM concentration); (FAM = 3' 5(6)-Carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine (quencher dye)); (example: use volumes below if stock primer is 10 µM concentration).

(e) PCR reactions:

The following real-time PCR reaction (or equivalent) should be set up in a clean environment. The reaction should be assembled using aerosol resistant pipette tips to decrease the chance of contamination. Total volume for reaction is 50µl (Opticon) or 25µl (SmartCycler).

Reaction Mix	50µl Volume	25µl Volume	Final Concentration
10X PCR Gold Buffer	5µl	2.5µl	1X
MgCl ₂ (25mM)	5µl	2.5µl	2.5mM
10mM dNTP mix	4µl	2.0µl	200µM each

Forward Primer (<i>sefAF</i>)	0.2µl	0.1µl	0.05uM
Reverse Primer (<i>sefAR</i>)	4.6µl	2.3µl	0.9 uM
TaqMan probe (<i>sefAPR</i>)	0.6µl	0.3µl	0.25uM
Amplitaq Gold Polymerase	0.25µl	0.13µl	1.25U
Sterile PCR Grade Water	25.35µl		10.17µl
DNA Template*	5.0µl	5.0µl	

*The DNA template is 5.0µl of the boiled MSRV solution or DNA from another DNA extraction method.

(f)PCR amplification program:

Pre-incubation	50°C for 2 minutes	1 cycle
Taq activation	95°C for 10 minutes	1 cycle
Annealing Step	95°C for 15 seconds	
Extension Step	60°C for 60 seconds	40 cycles

(g)Analysis of results:

A sample with a Ct value less than 35 is considered positive. A sample with a Ct value between 35 and 40 is considered to be indeterminate. Positive and indeterminate results are to be further tested by culture methods

Reference:

1. Seto KH, Valentin-Bon IE, Brackett, RE, and Holt, PS. (2004) Rapid, Specific Detection of Salmonella Enteritidis in Pooled Eggs by Real-Time PCR. Journal Food Protection: Vol 67, No. 5, pp. 864-869