FOOD SAFETY LABORATORY
CORNELL UNIVERSITY

PCR identification of serogroups for Salmonella isolates

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of the experiment is to identify common O-antigen types of *Salmonella* isolates using PCR.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

1.3 Definitions

Amplicon: a piece of DNA formed as the product of natural or artificial amplification events. For example, it can be formed via polymerase chain reactions (PCR) or ligase chain reactions (LCR), as well as by natural gene duplication.

Serogroup: a group of bacteria containing a common antigen, sometimes including more than one serotype, species, or genus. This is an unofficial designation, used in the classification of certain genera of bacteria, such as *Leptospira, Salmonella, Shigella*, and *Streptococcus*.

O-Antigen: The lipopolysaccharide-protein somatic antigens, usually from gram-negative bacteria, important in the serological classification of enteric bacilli. The O-specific chains determine the specificity of the O antigens of a given serotype. O antigens are the immunodominant part of the lipopolysaccharide molecule in the intact bacterial cell. (From Singleton & Sainsbury, Dictionary of Microbiology and Molecular Biology, 2nd ed)

Traditional or Immunological Serotyping: the process to determine serotypes based on serological method using a combination of antibodies which specifically recognize different antigens located in bacterial cell wall, flagella or capsule.

1.4 Safety

Salmonella is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with *Salmonella*. All waste from these experiments needs to be treated as BSL-2 waste.

SECTION 2 MATERIALS

- **dH2O.** Lab stock in media room.

- **10X AmpliTaq Gold® buffer.** Commercially purchased from Applied Biosystems. Kept in -20 ^{o}C.

- **25mM $MgCl_2$.** Commercially purchased from Applied Biosystems. Kept in -20 ^{o}C.

- **10 mM dNTPs.** Commercially purchased from Applied Biosystems. The original concentration is 100 mM, and needs to be diluted before use. Kept in -20 ^{o}C.

- **5 U/uL AmpliTaq Gold® DNA polymerase.** Commercially purchased from Applied Biosystems. Kept in -20 ^{o}C.

- **Primers.** Synthesized by IDT. The stock concentration is 100 uM, and the working concentration is 10 uM.

- **Genomic DNA or lysate of *Salmonella* isolates.**

Serogroup	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
B	GGC ATA TAT TTC TGT ATT CGC G	GCC TTA ATT AAG TAA GTT AGT GGA AGC	Herrera-Leon *et al.*, 2007
C1	CAG TAG TCC GTA AAA TAC AGG GTG G	CAA TGC TAT AAA TAC TGT GTT AAA TTG C	
C2-C3	ACT GAA GGT GGT ATT TCA TGG G	AAG ACA TCC CTA ACT GCC CTG C	
D	GAG GAA GGG AAA TGA AGC TTT T	TAG CAA ACT GTC TCC CAC CAT AC	
E1*	TAA AGT ATA TGG TGC TGA TTT AAC C	GTT AAA ATG ACA GAT TGA GCA GAG	
G	CTC TTG ATG AAT GTT ATT A	GTT AAC CCC TCC TAA TA	Fitzgerald *et al.*, 2007
K	CTC TAG GAT CAA CTG AAG GTG GTC	CAA CCC AGC AAT AAA GCA GAA	Fitzgerald *et al.*, 2006

SECTION 3 PROCEDURES

3.1 Multiplex PCR to determine D, B, C1, C2-C3, E1 Serogroups of *Salmonella*

1) In UV room (DNA Free) prepare Master Mix as below and dispense into PCR tubes;

Master Mix:	1X (25µl rxn vol):
dH_2O	14.375
10X AmpliTaq Gold® buffer	2.5
25 mM $MgCl_2$	1.5
B forward(10uM)	0.5
B reverse(10uM)	0.5
C1 forward(10uM)	0.5
C1 reverse(10uM)	0.5
C2 forward(10uM)	0.5
C2 reverse(10uM)	0.5
D forward(10uM)	0.5
D reverse(10uM)	0.5
E1 forward(10uM)	0.5
E1 reverse(10uM)	0.5
10mM dNTPs	0.5
AmpliTaq Gold® DNA polymerase	0.125
total	**24**

2) At the bench load 1 uL of DNA (standardized to 25 ng/uL preferred)- be sure to thaw DNA completely and mix w/ pipette;
3) Load PCR tubes into Thermalcycler and follow program below;

Temperature	Time	
95 C	10 min	
95 C	30 sec	30 cycles
58 C	30 sec	
72 C	45 sec	
72 C	7 min	
4 C	infinite	

4) Store PCR product at 4C until prepared to run gel;
5) Run product (B amplicon ~230 bp; C1 amplicon ~483 bp; C2-C3 amplicon ~154 bp; D amplicon ~615 bp; E amplicon ~345 bp) through 2% agarose gel at 120 V for 45 min;
6) Stain for ~30s in Ethidium Bromide;

7) Destain in water for minimum of 1 hour;
8) Visualize with UV light and take picture.

3.2 PCR to determine G Serogroup of *Salmonella*
1) In UV room (DNA Free) prepare Master Mix as below and dispense into PCR tubes;

Master Mix:	1X (50µl rxn vol):
dH_2O	16.875
10X AmpliTaq Gold® buffer	2.5
25mM MgCl2	1.5
10 uM F primer	1.25
10 uM R primer	1.25
10 mM dNTPs	0.5
AmpliTaq Gold® DNA polymerase	0.125
Total	**24**

2) At the bench load 1 uL of DNA (standardized to 25 ng/uL)- be sure to thaw DNA completely and mix w/ pipette;
3) Load PCR tubes into Thermalcycler and follow program below;

Temperature	Time	
95 C	10 min	
94 C	30 sec	30 cycles
58 C	30 sec	
72 C	30 sec	
72 C	7 min	
4 C	infinite	

4) Store PCR product at 4 C until prepared to run gel;
5) Run product (amplicon ~100bp) through 2-2.5% agarose gel (double with 1 comb) at 120 V for 45 min;
6) Stain for ~30s in Ethidium Bromide;
7) Destain in water for minimum of 1 hour;
8) Visualize with UV light and take picture.

3.3 PCR to determine K Serogroup of *Salmonella*
1) In UV room (DNA Free) prepare Master Mix as below and dispense into PCR tubes;

Master Mix:	1X (25µl rxn vol):
dH_2O	16.375
10X AmpliTaq Gold® buffer	2.5
25mM MgCl2	1.5
10 uM F primer	1.5
10 uM R primer	1.5

10 mM dNTPs	0.5
AmpliTaq Gold® DNA polymerase	0.125
total	**24**

2) At the bench load 1 uL of DNA (25 ng/uL preferred)- be sure to thaw DNA completely and mix w/ pipette;
3) Load PCR tubes into Thermalcycler and follow program below;

Temperature	Time	
95 C	10 min	
94 C	30 sec	
58 C	30 sec	30 cycles
72 C	45 sec	
72 C	10 min	
4 C	Infinite	

4) Store PCR product at 4C until prepared to run gel;
5) Run product (amplicon ~360 bp) through 2% agarose gel at 120 V for 30 min;
6) Stain for ~30s in Ethidium Bromide;
7) Destain in water for minimum of 1 hour;
8) Visualize with UV light and take picture.

SECTION 4 TROUBLESHOOTING

Problems previously encountered with this assay include:

1. Detection of serogroup F with C1 primers: if the H1 & H2 PCR detection results are consistent with that of traditional serotyping, then the positive C1 reaction may be regarded as F serogroup.
2. Detection of E4 with E1 primers: with the E1 primers, the isolates from E4 serogroup can also get positive result.
3. Pay attention to agarose concentration--some PCR products are small and can run off the gel quickly during electrophoresis at lower agarose concentrations
4. Do not be alarmed if molecular serotyping and traditional (immunological) results are not the same-- traditional serotyping can be incorrect (may only be ~80% reliable)

Observation	**Possible Cause(s)**	**Solution(s)**
No amplification Product	Poor primer design	Verify that primers are non-complementary, both internally and to each other. Increase length of primer.
	Poor primer specificity	Verify that oligos are complementary to proper target sequence.
	Insufficient primer concentration	Increase primer concentration to 0.1–0.5 µM.
	Missing reaction component	Repeat reaction setup.
	Target sequence not present in template DNA	Try other sources of template DNA.
	Poor reaction conditions	Optimize (Mg++), annealing temperature and extension time. Thoroughly mix Mg++ solution. Check primer concentrations.
	Questionable template quality	Analyze DNA via gel electrophoresis after incubation with Mg^{++}.
	Inhibitory substance in reaction	Decrease sample volume. Try alcohol precipitation or drop dialysis to further purify DNA.
	Insufficient number of cycles	Return reaction to thermocycler and run additional cycles.
	Incorrect thermocycler programming	Check program, verify times and temperatures.
	Inconsistent block temperature	Test calibration of heating block.
	Reaction tubes or solutions contaminated	Autoclave tubes prior to use to eliminate biological inhibitors.
Multiple or non-specific products	Premature *Taq* DNA Polymerase replication	Set up reactions on ice with chilled components. Add samples to pre-heated (95°C) thermocycler.

	Primer annealing temperature too low	Raise annealing temperature in 2°C increments.
	Insufficient mixing of reaction buffer	Reaction buffer must be thoroughly mixed.
	Improper Mg^{++} concentration	Adjust Mg^{++} concentration in 0.5 mM increments.
	Poor primer design	Verify that primers have no complementary regions – either internally or to each other. Try longer primers. Avoid GC-rich 3´ ends.
	Excess primer	Reduce primer concentration to 0.1–0.5 μM.
	Contamination with exogenous DNA	Use positive displacement pipettes or non-aerosol tips. Set-up dedicated work area and pipettor for reaction setup. Wear gloves during reaction setup.
	Multiple target sequences in template DNA	Redesign primers with higher specificity to target sequence.
Clones contain mutations	Excessive Mg^{++}	Use minimal concentration of Mg^{++} to produce desired amount of product.
	Wild-type target sequence may be toxic to host	Use low-copy cloning vector.Clone into non-expression vector.

SECTION 5 REFERENCES

Herrera-Leon et al. (2007). Blind comparison of traditional serotyping with three multiplex PCRs for the identification of Salmonella serotypes. Res Microbol, 158(2):122-7.

Fitzgerald et al. (2007). Multiplex, bead-based suspension array for molecular determination of common Salmonella serogroups. J Clin Microbiol, 45(10):3323-34.

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