PCR determination of H-antigens for Salmonella isolates

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of the experiment is to determine H-antigen (H1 and H2) types of *Salmonella* isolates combined with PCR amplification and DNA sequencing.

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

H antigen: flagellar antigen, any of various antigens associated with the flagella of motile bacteria and used in serological identification of enteric bacilli.
H1 antigen: phase-1 flagellar antigen, encoded by the *fliC* gene.
H2 antigen: phase-2 flagellar antigen, encoded by the *fljB* gene, some bacterial serotypes are phase-2 negative.
Amplicon: a strand of DNA formed as the product of natural or artificial amplification events. In the scope of this protocol, it is formed via polymerase chain reactions (PCR).
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.

- **1 UN/ul Shrimp Alkaline Phosphatase.** Commercially purchased from USB Corp. Kept in -20 ^{o}C.

- **10 UN/ul Exonuclease I.** Commercially purchased from USB Corp. Kept in -20 ^{o}C.

- **Primers.** Synthesized by IDT. The stock concentration is 100 uM, and the work concentration is 10 uM except that of MR-1/MR-2 is 1 uM.(used for sequencing only)

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

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
H1	ATG GCA CAA GTC ATT AAT AC	TTA ACG CAG TAA AGA GAG GAC	Mortimer *et al.* 2004
H2	GGCACAAGTAATCAACACTAACA	CATTTACAGCCATACATTCCATA	FSL
Sequencing	AAC AAC AAC CTG CAG CGT GTG	GTC GGA ATC TTC GAT ACG GCT AC	FSL

SECTION 3 PROCEDURES

3.1 PCR to determine H1 type 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 360 buffer	2.5
25mM MgCl2	2
10 uM FL_START2	1
10 uM rFSa1	1
10 mM dNTPs	1
Taq gold (5u/uL)	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;

95 C - 10 min	
95 C - 30 sec 59 C - 30 sec 72 C - 90 sec	20 cycles,-0.5 C/cycle
95 C - 30 sec 49 C - 30 sec 72 C - 90 sec	20 cycles
72 C - 7 min	
4 C infinite	

4) Store PCR product at 4C until prepared to run gel;
5) Run PCR product through 1.5% agarose gel at 120 V for 25-30 min; amplicon ~1300-1500bp;
6) Stain for ~30s in Ethidium Bromide;
7) Destain in water for minimum of 1 hour;
8) Visualize with UV light and take picture;
9) If the band on gel is specific, clear and brilliant without primer dimers, add 1 ul of Exonuclease I (EXO) and 1 ul of Alkaline phosphatase (SAP) into each tube containing the rest PCR products, use the following thermalcycler program;

37 C - 45 min
80 C -15 min
4 C -infinite

10) Mix 8 ul of 1 uM MR-1 or MR-2 primers with 10 ul purified PCR products, send for double coverage DNA sequencing of *fliC* gene;

11) When DNA sequencing ran well, edit the sequences and Blast it to search the best matched *fliC* sequences in GenBank.

3.2 PCR to determine H2 type 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 360 buffer	2.5
25mM MgCl2	1.5
10 uM MR-22 fljBF	1
10 uM MR-23 fljBR	1
10 mM dNTPs	1
Taq gold (5u/uL)	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;

95 C - 10 min	
95 C - 30 sec 58 C - 30 sec 72 C - 90 sec	30 Cycles
72 C - 7 min	
4 C – inifinite	

4) Store PCR product at 4 C until prepared to run gel;

5) Run PCR product through 1.5% agarose gel at 120 V for 25-30 min; amplicon ~1600bp;

6) Stain for ~30s in Ethidium Bromide;

7) Destain in water for minimum of 1 hour;

8) Visualize with UV light and take picture;

9) Some isolates are H2 antigen negative, therefore no amplicon will be observed; if the band on gel is specific, clear and brilliant without primer dimers, add 1 ul of Exonuclease I (EXO) and 1 ul of Alkaline phosphatase (SAP) into each tube containing the PCR products, use the following thermalcycler program;

37 C - 45 min
80 C -15 min
4 C -infinite

10) Mix 8 ul of 1 uM MR-1 or MR-2 primers with 10 ul purified PCR products, send for double coveraged DNA sequencing of *fljB* gene;

11) When DNA sequencing ran well, edit the sequences and Blast it to search the most matched *fljB* sequences in GenBank.

SECTION 4 TROUBLESHOOTING

Problems previously encountered with this assay include:

1. Some Salmonella strains lack the H2 antigen, so a PCR may not always produce an amplicon
2. *fliC* amplicon is variable- the size can range from 1300-1600bp

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

Mortimer et al. (2004). Towards the development of a DNA-sequence based approach to serotyping of Salmonella enterica. BMC Microbiology, 4:31.