FOOD SAFETY LABORATORY
CORNELL UNIVERSITY

***inlA* ORF & Gene Fragment PCR & Sequencing**

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SECTION 1 INTRODUCTION

1.1 Purpose

To provide a standard laboratory procedure for PCR and subsequent sequencing of the entire inlA ORF.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases and any experimental procedures conducted by laboratory members at other locations.

1.3 Definitions

SECTION 2 MATERIALS

***inlA* gene PCR Primer Sets:**

The whole inlA ORF is amplified as four fragments using the four pairs of primers shown.

Primer	Sequence (5'–3')	Annealing temp. (°C)	Amplicon size
inlA proF inlA proR	TTT TAA AAG GTG GAA TGA CA GAA GCG TTG TAA CTT GGT CTA	52	500 bp
inlA F1 inlA S1R	CAG GCA GCT ACA ATT ACA CA GGA CTG ATG TTA CTT ATT TGG T	52	800 bp
inlA F2 inlA S2R	AAG ATA TAG GCA CAT TGG CGA GTT CGT ACT GAA ATY CCA KTT AGT TCC	57	800 bp
inlA seq F inlA R	GTG GAC GGC AAA GAA ACA AC ATA TAG TCC GAA AAC CAC ATC T	52[a]	900 bp

Exact primer locations outlined on next page of SOP

When amplifying and sequencing to determine the presence and location of Premature Stop Codons (PMSC) start with the 3' end of the inlA ORF. Locations of PMSCs are contained within the papers listed under this SOPs references section.

L. monocytogenes EGD-e|LMO00433|inlA: 2403 bp - Internalin A

agttttggcggtaagagtgcggggcttgaattcgtcaataatagaccattttaaaaggtggaatgacacgtgtgatat (inlA-proF)
caacataaaatgatttgcatgattgaattcctattaagcttcgatagtgaaatacataaaaatattatatataggaaaaat
gtgctggaacgaacgagatgaaggatatcactaaacggctccgtagacagattagctactaaataccataggaac
aattcgtggggagcataatttaataaattaagcagacatgatttttcggatgcaggagaaaatcctatacaacgaaac
ctgatattgtttctaactattgaaaaaggagtgtatata

1 - gtg aga aaa aaa cga tat gta tgg ttg aaa agt ata cta gta gca ata tta gta ttt ggc
61 - agc gga gta tgg att aac acg agt aac ggg aca aat gct cag gca gct aca att aca caa (inlAF1)
121 - gat act cct att aat cag att ttt aca gat aca gct cta gcg gaa aaa atg aag acg gtc
181 - tta gga aaa acg aat gta aca gac acg gtc tca caa aca gat cta gac caa gtt aca acg (inlA-proR)
241 - ctt cag gcg gat aga tta ggg ata aaa tct atc gat gga gtg gaa tac ttg aac aat tta
301 - aca caa ata aat ttc agc aat aat caa ctt acg gac ata acg cca ctt aaa aat tta act
361 - aag tta gtt gat att ttg atg aat aat aat caa ata gca gat ata act ccg cta gct aat
421 - ttg acg aat cta act ggt ttg act ttg ttc aac aat cag ata acg gat ata gac ccg ctt
481 - aaa aat cta aca aat tta aat cgg cta gaa cta tcc agt aac acg att agt gat att agt
541 - gcg ctt tca ggt tta act agt cta cag caa tta tct ttt ggt aat caa gtg aca gat tta
601 - aaa cca tta gct aat tta aca aca cta gaa cga cta gat att tca agt aat aag gtg tcg
661 - gat att agt gtt ctg gct aaa tta acc aat tta gaa agt ctt atc gct act aac aac caa
721 - ata agt gat ata act cca ctt ggg att tta aca aat ttg gac gaa tta tcc tta aat ggt
781 - aac cag tta aaa gat ata ggc aca ttg gcg agt tta aca aac ctt aca gat tta gat tta (inlAF2)
841 - gca aat aac caa att agt aat cta gca cca ctg tcg ggt cta aca aaa cta act gag tta
901 - aaa ctt gga gct aac caa ata agt aac atc agt ccc cta gca ggt tta acc gca ctc act (inlAS1R)
961 - aac tta gag ctt aat gaa aat cag ctg gaa gat att agc cca att tct aac ctg aaa aat
1021 - ctc aca tat tta act ttg tac ttt aat aat ata agt gat ata agc cca gtt tct agt tta
1081 - aca aag ctt caa aga tta ttt ttc tat aat aac aag gta agt gac gta agc tca ctt gcg
1141 - aac tta aca aat att aat tgg ctt tca gct ggg cat aac caa att agc gat ctt aca cca
1201 - ttg gct aat tta aca aga atc acc caa cta ggg ttg aat gat caa gca tgg aca aat gca
1261 - cca gta aac tac aaa gca aat gta tcc att cca aac acg gtg aaa aat gtg act ggc gct
1321 - tta att gca cca gct act att agc gat ggc ggt agt tac aca gag cct gat ata aca tgg
1381 - aac tta cct agt tat aca aat gaa gta agc tat acc ttt agc caa cct gtc act att gga
1441 - aaa gga acg aca aca ttt agt gga acc gtg acg cag cca ctt aag gca att ttt aat gtt
1501 - aag ttt cat gtg gac ggc aaa gaa aca acc aaa gaa gtg gaa gct ggg aat tta ttg act (inlAseqF)
1561 - gaa cca gct aag ccc gta aaa gaa ggt cac aca ttt gtt ggt tgg ttt gat gcc caa aca
1621 - ggc gga act aaa tgg aat ttc agt acg gat aaa atg ccg aca aat gac atc aat tta tat (inlAS2R)
1681 - gca caa ttt agt att aac agc tac aca gca acc ttt gat aat gac ggt gta aca aca tct
1741 - caa aca gta gat tat caa ggc ttg tta caa gaa cct acg gca cca aca aaa gaa ggt tat
1801 - act ttt aaa ggc tgg tat gac gca aaa act ggt ggt gac aag tgg gat ttc gca act agc
1861 - aaa atg cct gct aaa aac atc acc tta tat gcc caa tat agc gcc aat agc tat aca gca
1921 - acg ttt gat gtt gat gga aaa tca acg act caa gca gta gac tat caa gga ctt cta aaa
1981 - gaa cca aag gca cca acg aaa gcc gga tat act ttc aaa ggc tgg tat gac gaa aaa aca
2041 - gat ggg aaa aaa tgg gat ttt gcg acg gat aaa atg cca gca aat gac att acg ctg tac
2101 - gct caa ttt acg aaa aat cct gtg gca cca cca aca act gga ggg aac aca ccg cct aca
2161 - aca aat aac ggc ggg aat act aca cca cct tcc gca aat ata cct gga agc gac aca tct
2221 - aac aca tca act ggg aat tca gcc agc aca aca agt aca atg aac gct tat gac cct tat
2281 - aat tca aaa gaa gct tca ctc cct aca act ggc gat agc gat aat gcg ctc tac ctt ttg
2341 - tta ggg tta tta gca gta gga act gca atg gct ctt act aaa aaa gca cgt gct agt aaa
2401 - tag

aagtagtgtaaagagctagatgtggttttcggactatatctagctttttattttttaataactagaatcaaggagag (inlAR1)
gatagtgtgaaagaaaagcacaacccaagaaggaagtattgtttaatctcaggtttagctattatttttagtttatg
gataattattggaaacggggcgaaagtacaagcggagactatcaccgtgccaacgccaatcaagcaaatttttt
tcagatgatgcttttgcagaaacaatcaaagacaatttaaagaaa

SECTION 3 PROCEDURES

1. PCR amplification using primers inlA proF and inlA proR

1A.Master Mix preparation:

PCR Reagents [Concentration]	Volume (µl) for 1 reaction
inlA proF [12.5µM]	2
inlA proR [12.5µM]	2
DNTPs [10mM]	1
$MgCl_2$ [25mM]	3
Flexi 5X PCR buffer	10
Taq DNA polymerase	0.25
dH_20	30.75
TOTAL	49

1B. Thermocycling conditions:

PCR cycling conditions:

3 minutes	at	94° C	
1 minute	at	94° C	
1 minute	at	52° C	35X
1 minute	at	72° C	
5 minutes	at	72° C	
∞	at	4° C	

2. PCR amplification using primers inlA F1 and inlA S1R

2A. Master Mix preparation:

PCR Reagents [Concentration]	Volume (µl) for 1 reaction
inlA F1 [12.5µM]	2
inlA S1R [12.5µM]	2
DNTPs [10mM]	1
$MgCl_2$ [25mM]	3
Flexi 5X PCR buffer	10
Taq DNA polymerase	0.25
dH_20	30.75
TOTAL	49

2B. Thermocycling conditions:

PCR cycling conditions:

3 minutes	at	94° C	
1 minute	at	94° C	
1 minute	at	52° C	35X
1 minute	at	72° C	
5 minutes	at	72° C	
∞	at	4° C	

3. PCR amplification using primers inlA F2 and inlA S2R

3A. Master Mix preparation:

PCR Reagents [Concentration]	Volume (µl) for 1 reaction
inlA F2 [12.5µM]	2
inlA S2R [12.5µM]	2
DNTPs [10mM]	1
$MgCl_2$ [25mM]	3
Flexi 5X PCR buffer	10
Taq DNA polymerase	0.25
dH_20	30.75
TOTAL	49

3B. Thermocycling conditions:

PCR cycling conditions:

3 minutes	at	94° C	
1 minute	at	94° C	
1 minute	at	57° C	35X
1 minute	at	72° C	
5 minutes	at	72° C	
∞	at	4° C	

4. PCR amplification for primers inlA seq F and inlA R.

This is a "hot start PCR". The taq polymerase (added to a separate mix) is initially withheld from the reaction tubes and is not added until the thermocycler enters the first cycle and drops from 94°C to 80°C. The machine is to be paused at 80°C to add hot start mix to each tube. Once this is done, the PCR cycling can be resumed.

4A. Master Mix preparation:

PCR Reagents [Concentration]	Volume (µl) for 1 reaction
inlA seq F [12.5µM]	2
inlA-R [12.5µM]	2
DNTPs [10mM]	1
$MgCl_2$ [25mM]	3
Flexi 5X PCR buffer	8
dH_20	28
TOTAL	44

44 µl of Master mix per 0.2ml tube + 1 µl of **Lysate/template DNA**

Hot Start Taq® Mix preparation

PCR Reagents [Concentration]	Volume (µl) for 1 reaction
Flexi 5X PCR buffer	2
Taq DNA polymerase	0..25
dH_20	3

5 µl of Hot Start mix per 0.2ml tube add at 80^o C hold during PCR cycling.

The reaction and hot start mixes above are for a 50 µl final volume.

4B. Thermocycling conditions:

PCR cycling conditions:

3 minutes	at	94° C	
1 minute	at	94° C	
1 minute	at	52° C	35X
1 minute	at	72° C	
5 minutes	at	72° C	
∞	at	4° C	

Purification & Quantification of PCR fragments.

1. Confirm clean amplification of each PCR product by gel electrophoresis alongside a DNA ladder to ensure correct amplicon size.
2. Purify amplicons using commercially available kits or reagents (i.e. Qiaquick® 8 PCR Purification Kit from Qiagen or Exo-SAP procedure).
3. Quantify the DNA using a suitable available method (e.g. gel electophoresis, Nanodrop system, Hoechst dye etc.)
   Note: If using the Exo-SAP purification protocol, quantification of DNA is unnecessary.

Sequencing

Consult your sequencing facility's requirements for sequencing submissions. Send the appropriate amount of DNA with the appropriate concentration of one primer per sequencing reaction tube. If you amplified your DNA with the forward (inlA seq F_1) and reverse (inlA-R_1) primers then that amplicon must be submitted for sequencing with the same primers.

SECTION 4 REPORTING and LABELING

All procedures and results need to be reported in a lab book with dates. Sequencing files are to be maintained in a single sequencing folder organized into subfolders by fragment and further subdivided into raw files, contigs, finished seqs, etc.

SECTION 5 TROUBLESHOOTING

SECTION 6 REFERENCES

Select Listeria monocytogenes subtypes commonly found in foods carry distinct nonsense mutations in inlA, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. Nightingale KK, Windham K, Martin KE, Yeung M, Wiedmann M. Appl Environ Microbiol. 2005 Dec;71(12):8764-72.

Development and implementation of a multiplex single-nucleotide polymorphism genotyping assay for detection of virulence-attenuating mutations in the Listeria monocytogenes virulence-associated gene inlA. Van Stelten A, Nightingale KK. Appl Environ Microbiol. 2008 Dec;74(23):7365-75.