Molecular Subtyping of *Listeria monocytogenes* and *Listeria species* by Pulsed field Gel Electrophoresis (PFGE)

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SECTION 1 INTRODUCTION

1.1 Purpose: This protocol describes the standardized method of molecular typing by PFGE of *Listeria monocytogenes* and *Listeria species* as to be used in the FSL laboratory. This SOP is a recreation of the standardized CDC Pulsenet protocol (original PulseNet protocol can be found under the reference section of this SOP).

1.2 Scope: This procedure is used for molecular typing by PFGE of *Salmonella spp, E.coli* and *Shigella sonnei* and *Shigella flexneri* under the supervision of Martin Wiedmann within the department of Food Science at Cornell University.

1.3 Definitions

PFGE: Pulsed Field Gel Electrophoresis
BHI: Brain Heat Infusion Agar
CSB: Cell Suspension Buffer
CLB: Cell Lysis Buffer
SKG: SeaKem Gold Agarose
PK: Proteinase K

SECTION 2 MATERIALS

1M Tris HCl, pH 8.0
0.5M EDTA, pH 8.0
TE Buffer, pH 8.0
Cell Suspension Buffer (CSB)
Cell Lysis Buffer (CLB)
10X TBE, pH 8.0
0.5X TBE
UP H20 (autoclaved)
20% SDS
Proteinase K (Roche Applied Science)
Lysozyme (Sigma-Aldrich BioUltra)
RCT
GCP
Loops
Sticks
Cotton swabs
Razor

37C, 55-60C, 25C waterbaths
Spatula
Sterile 125 mL bottles
1.5 mL Eppendorf tubes
50 mL conical tube
Plug Mold (BioRad)
Screened caps (BioRad)
Gel Mold & Frame (BioRad)
Restriction Buffer A (Roche Applied Sciences)
Restriction Enzyme *Apa1* (40U/uL, Roche Applied Sciences)
Restriction Buffer NEB4 (New England Biolabs)
Restriction Enzyme *Asc1* (10U/uL, New England Biolabs)
100X BSA (New England Biolabs)

SECTION 3 PROCEDURE

Overview of Typical Work Flow:

Day 1: Grow up isolates overnight
Day 2: Plug Prep & Washing
Day 3: Restriction Digest
Day 4: Gel Run
Day 5: Gel Staining & Photographing Gel Image

1. Streak isolates for overnight growth:

***Note:** Before working with BSL-2 organisms please review proper handling techniques.

1. Using a sterile stick, streak out isolate(s) from glycerol stock onto a BHI plate. Streak a heavy lawn as you will need many cells for cell suspension preparation in the next step of protocol.
2. Allow plate to grow in 37C incubator overnight (12-18 hours). *Note: Always use freshly streaked plates for preparation of cell suspensions as cells are easier to lyse. Overnight growth should be no older than 18 hours.
3. Do not forget you will need to prepare *S. braenderup* reference plugs (FSL W3-083) for gel runs. Reference plugs are made in the same manner all other *Salmonella* plugs are made; refer to “Salm_PFGE_SOP”.

2. Plug Preparation:

1. Turn on Spectrophotometer, set wavelength to 610nm, and allow to warm up for ~15 minutes. Blank spectrophotometer with 3 mL of TE in a GCT.
2. Turn on 55-60C waterbath. Place 20%SDS in 55-60C waterbath to prewarm.
3. Turn on 37C waterbath.
4. For each isolate: label a RCT (these will be used to prepare cell suspensions), a 1.5mL epp tube, and a 50mL conical tube.
5. Fill RCTs with 3.5 mL TE. Fill twice as many GCTs with 2.0 mL TE (these will be used for altering concentration of your cell suspensions and measuring the ODs)
6. Using a sterile cotton swab that has been premoistened with TE, remove cells from the lawn of the BHI plate and suspend the cells in the TE RCT by gently swirly sterile swab.
7. Gently pipette up and down the cell suspension to achieve a homogeneous suspension of cells and transfer 1.0 mL of this suspension into a GCT containing 2.0 mL TE.
8. Measure the OD of the GCT cell suspension. Target OD range is 0.340-0.430.
9. After achieving an OD within the target range, store RCT cell suspensions on ice. Repeat for all isolates.
10. Distribute 400 uL of adjusted cell suspensions into labeled 1.5mL epp tubes and incubate in 37C waterbath for 10 minutes.
11. Prepare 20mg/mL Lysozyme Soln:
    0.013g Lysozyme + 650 uL TE
12. Remove cell suspensions from 37C waterbath and add 20 uL of the Lysozyme solution to each tube and incubate tubes in 55-60C waterbath for 20 minutes. *Note: Some strains that exhibit endogenous nuclease activity after lysis, better quality patterns can be achieved by decreasing the lysing incubation time to ≤10 minutes.
13. Prepare 20mg/mL Proteinase K Soln (to be used in plug prep and in lysing solution):
    0.013g PK + 650 uL UP H20 (enough for the preparation of 12 isolates; will need 45 uL PK soln. per isolate)
14. Prepare 1.0% SKG Agarose in sterile bottle or flask:
    0.25g SKG + 23.5mL TE Buffer → Microwave until completely dissolved (about 1-2 minutes) → Add 1.25 mL 20%SDS to melted agarose and swirl gently to mix. *Note: If floppy or easily damaged plugs are produced, increase microwave time.
15. Remove cell suspensions from 55-60C waterbath and add 20 uL of the PK solution to each tube.
16. Mix 400 uL of the SKG/SDS soln with the entire cell suspension/PK soln and rapidly mix by pipetting or vortexing (to avoid agarose solidification) and immediately cast into cleaned plug mold.

17. Prepare lysing solution mastermix in sterile bottle:
    n= # of isolates
    5 mL CLB x (n+1)
    25 uL PK x (n+1)
18. Distribute 5 mL of lysing solution mastermix into each 50 mL conical tube.
19. Using cleaned spatula, transfer solidified plugs into corresponding conical tube.
20. Lyse plugs in conical tubes in shaker incubator at 54C/ 170RPM for 3.5-16 hours. *Note: CDC protocol lyses for 1.5-2 hrs. FSL has found better lysis of cells is achieved after a minimum of 3.5 hours.

3. Plug Washing:

1. After plugs have been lysed, remove conical lid and replace with screen caps.
2. Drain lysing solution from plugs and replace with 10 mL autoclaved UP H20. Return plugs to shaker incubator for 10 minutes at 50C/ 70RPM.
3. Drain and repeat one time (2 total water plug washes)
4. Drain and replace with 10 mL TE Buffer. Return to shaker incubator for 15 minutes at 50C/ 70 RPM.
5. Drain and repeat three more times (4 total TE Buffer plug washes).
6. Drain last wash and store in 5 mL TE at 4C until proceeding to next step of protocol. *Note: Plugs stored this way can be good for years.

4. Restriction Digest of Agarose Slices (*Asc1* & *Apa1*):

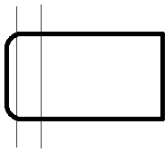
1. Label two 1.5 mL epp tubes for each isolate
2. Prepare buffer solutions:

	Amount/ plug slice	x (n+1)	Amount in Mastermix:
Autoclaved UP H20	135 uL		
Restriction Buffer A	15 uL		

	Amount/ plug slice	x (n+1)	Amount in Mastermix:
Autoclaved UP H20	180 uL		
Restriction Buffer NEB4	20 uL		

3. Distribute 150 uL of restriction buffer soln A into each epp tube.
4. Distribute 200 uL of restriction buffer soln NEB4 into each epp tube.
5. Using cleaned razor and spatula, cut rounded bottom edge of plug off and discard. Cut two ~2mm slices from the plug (as depicted below) and place

one in Buffer A soln and the other in Buffer NEB4 soln.



*Note: The shape of your plug slice will determine the shape of your resulting pattern, so it is important to cut a clean sharp rectangle.

6. Incubate Buffer A slices in 25C waterbath for 10-15 minutes.
7. Incubate Buffer NEB4 slices in 37C waterbath for 10-15 minutes.
8. Prepare restriction enzyme solutions:

	Amount/ plug slice	x (n+1)	Amount in Mastermix:
Autoclaved UP H20	130 uL		
Restriction Buffer A	15 uL		
Restriction Enzyme *Apa1* (40U/uL)	1.25 uL		
100X BSA	2 uL		

	Amount/ plug slice	x (n+1)	Amount in Mastermix:
Autoclaved UP H20	175.5 uL		
Restriction Buffer NEB4	20 uL		
Restriction Enzyme *Acs1* (10U/uL)	2.5 uL		
100X BSA	2 uL		

9. Aspirate off buffer soln from plug slices and replace with 200 uL *Asc1* enzyme solution or 150 uL *Apa1* enzyme solution.
10. Return slices to respective waterbaths for 3-7 hours.
    *Note: Digestion time can be shortened; however, “shadow bands” or incomplete digestion may be observed.
11. After digestion, aspirate off enzyme soln. on slices and replace with 200 uL TE Buffer and store in 4C until next step of the protocol.

5. Gel preparation and gel run:

1. Turn on 55-60C waterbath.
2. Prepare 1% SKG gel in sterile flask:

	10 well gel	15 well gel
SKG	1.0 g	1.5 g
10X TBE	5.0 mL	7.5 mL
UP H2O	95.0 mL	142.5 mL

3. To avoid additional water loss in the microwave process, weigh gel flask and zero scale. Add 8 mL autoclaved UP H2O and microwave. Microwave gel flask until these additional 8.0 mL of water has evaporated and scale returns to zero.
4. Pour gel into cleaned gel mold. Save last 1.5- 2.0 mL of gel for sealing the wells in an epp tubes. Keep extra gel from solidifying by placing in 55-60C waterbath. Do not let gel to stand for a long time before pouring as this will result in a "swirly" effect in the solidified gel.
5. Cover gel and gel mold with tupperware container to eliminate dust.
6. Allow gel to solidify for 35-40 minutes.
7. Remove digested plug slices from 4C and aspirate off TE Buffer. Replace with 200 uL of 0.5X TBE and let stand at RT for ~15 minutes.
8. Using cleaning spatula, remove slices from epp tubes and blot slice gently with Kimwipe to remove excess TE Buffer and load into well. *Note: When loading slices it is important to not damage/ crush slices, load them uneven within well, or create air bubbles in wells. All these will result in interruptions in resulting patterns
9. If using 10 well gel, lanes 1 and 10 should be used for reference slices. If using 15 well gel, lanes 1, 8, and 15 should be reference slices.
10. When all slices are loaded, seal slices into wells by slowing pipetting saved agarose from step #4 onto slices.

6. Staining and Photographing Gel:

1. Turn on GelDoc system and open the Quantity One software.
2. Open Live image by selecting File > GelDoc XR.
3. Center gel within grid. It is important to center gel as this will be the final image uploaded into BioNumerics for analysis.
4. Manually Acquire image. A good starting exposure time should be ~0.500. Adjust exposure and focus accordingly.
5. Save image and Print copy for your lab notebook.
6. Once the gel picture has been captured, the file must be exported into a TIFF image file that BioNumerics recognizes.
7. Export into TIFF image: File > Export to TIFF image > Under Publishing, Export view excluding overlays" > Under Resolution, Same as Scan > Export
8. For information regarding processing gel fingerprint and labeling in BioNumerics, refer to SOP entitled "CU Naming SOP".

SECTION 4 REPORTING and LABELING

All data about isolates, PFGE protocol & steps, amount of reagents used, and gel results (including a picture of the gel) should be kept in your official lab notebook.

SECTION 5 TROUBLESHOOTING

Problems previously encountered with PFGE include:

(1) Smeary patterns usually results from exonuclease contamination in the plug itself or in the digestion reaction. Be careful as to not touch the plug to anything not sterilized or that has not been cleaned with ethanol (i.e. Razor blades, tubes, spatula, etc.)
(2) Poor pattern quality; incomplete digest/ partials: Use BSA in all digestion reactions; Incubate for longer periods of time (4-6 hrs); not optimal digestion reaction: check digestion calculations and reagents.

Rationales for modifications to the CDC PulseNet protocol:
(1) Increased OD yields brighter/ more intense bands even at low MW
(2) Increased plug lysis time yields better lysis of plugs, therefore resulting in better quality patterns.
(3) Changes to digestion reaction mixtures were based on old CDC PulseNet PFGE protocols and we have found original recipes produce better patterns.
(4) In some instances, enzymes are ordered in high concentration as they are more cost effective. However, number of units per digestion rxn/ slice remains constant.
(5) Digest durations have been extended to decrease likelihood of partial/ incomplete digests.

ADDITIONAL HELP

Below is a link for additional trouble-shooting help made available by PulseNet International:

http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/PFGE_troubleshooting.pdf

SECTION 6 REFERENCES

1. http://pulsenetinternational.org/downloads/pfge/5.3_2009_PNetStandProtLMonocytogenes.pdf
2. **Graves, L.M., B. Swaminathan.** 2001. PulseNet standardization protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int J Food Micro. 65: 55-62.