# Standardized Protocol For Molecular Subtyping of *Listeria monocytogenes* by Pulsed-Field Gel Electrophoresis (PFGE)

**BIOSAFETY WARNING**: The infectious dose for listeriosis has not been determined and it may depend, in part, on the susceptibility of the host. Groups at highest risk of acquiring infection are pregnant women, neonates, immunocompromised patients, and the elderly, although up to 30% of adults with listeriosis may be immunocompetent. Therefore, laboratorians working with *Listeria monocytogenes*, particularly those who may be at increased risk of acquiring listeriosis, should be made aware of this potential and advised to be particularly cautious when working with this organism.

**Please read laboratory instructions before continuing.** Treat all plastic ware, glassware, pipettes, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect, according to instructions. **Prepare all reagents and stock solutions prior to starting protocol (see Section 5a).**

## 5.30 Cultures/Cell Suspension

1. Grow bacteria on Brain Heart Infusion Agar (BHIA) plates in a 37ºC incubator overnight. Using a cotton swab, remove bacteria from the plates to tubes (Falcon 2057, 14 ml -17 x 100 mm) containing 3 milliliters (ml) of TE.

**Note: Thaw lysozyme stock (10 mg/ml) solution and proteinase K stock (20 mg/ml) solution, keep on ice. Discard any leftover (thawed) lysozyme or proteinase K stock solutions after step 5.34.**

2. Using a spectrophotometer, adjust the optical density of bacterial cell suspensions to 1.3 (range of 1.25 to 1.35) at 610 nm with TE. **If using the MicroScan Turbidity Meter (Dade International, Inc.), the equivalent range is 0.79 - 0.82 (for cell suspensions in Falcon 2057 tubes).** The graduated marking on the Falcon 2057 tube should face the front of the tube to avoid deflecting the light path.

3. Transfer 240 µl of each bacterial suspension to a 1.5 microcentrifuge tube.

4. Add 60 µl of **Lysozyme solution** (10 mg/ml) and mix by pipetting up and down. **Do not vortex**.

5. Incubate in a water bath at 37ºC for **10 minutes**.

## 5.31 Preparation of SSP (1.2% SeaKem Gold:1% Sodium Dodecyl Sulfate: 0.2 mg/ml proteinase K).

1. Prepare 10 ml of 1.2% SeaKem Gold agarose in sterile Type 1 water by dissolving 0.12 g of SeaKem Gold agarose in 10 ml of Type 1 water in a 125 ml screw cap Wheaton bottle or flask. Microwave the agarose until it completely melts; keep in a **53º to 56ºC** water bath.

2. Prepare the volume of SSP solution needed according to Table 5.31a in the following order:

   a. Add 10% SDS to a 50 ml polypropylene screw-cap tube, then place the tube in a beaker containing water at **53º to 56ºC**.

   b. Add 1.2% SeaKem Gold Agarose (from step 1) to the tube containing 10% SDS, mix by swirling while keeping the tube in warm water (**53º to 56ºC**).

   c. Add proteinase K just before ready to use; mix well by swirling while keeping the tube in warm water (**53º to 56ºC**).

Table 5.31a

| Number of strains | SDS (10%) | SeaKem Gold Agarose (1.2%) | Proteinase K (20 mg/ml) |
|---|---|---|---|
| 1 | 30 µl | 267 µl | 3 µl |
| 11 | 330 µl | 2.9 ml | 33 µl |
| 13 | 390 µl | 3.5 ml | 39 µl |
| 16 | 480 µl | 4.3 ml | 48 µl |
| 25 | 750 µl | 6.7 ml | 75 µl |

**5.32 Plug preparation**.

1. Remove cell/lysozyme suspensions in 1.5 ml tubes from 37ºC water bath (5.3, step 5).

2. Add 300 µl of the warm (**53º to 56ºC**) SSP solution to 300 µl cell suspensions; mix by gently pipetting mixture up and down a few times.

3. Immediately, dispense mixture into wells in plug molds. Do not allow bubbles to form. Allow plugs to solidify for 10-15 minutes at room temperature. Alternately, plugs may be solidified by placing in a refrigerator for 5 minutes.

**Note: When reusable plug molds (2-cm x 1-cm x 1.5-mm) are used, up to 2 plugs can be made from these amounts of cell suspe nsion and agarose; when disposable plug molds (1.5 mm x 10-mm x 5-mm) are used three to four plugs can be made.**

**5.33 Lysis**

1. Prepare cell lysis buffer (50 mM Tris pH 8.0, 50 mM EDTA, pH 8.0, 1% Sarkosyl, 0.15 mg/ml proteinase K)

according to Table 5.33a.

Table 5.33a

| Number of samples | 50 mM Tris pH 8.0, 50 mM EDTA, pH 8.0, 1% Sarkosyl | Proteinase K (20 mg/ml) |
|---|---|---|
| 1 | 4 ml | 30 µl |
| 10 | 40 ml | 300 µl |
| 13 | 52 ml | 390 µl |
| 15 | 60 ml | 450 µl |
| 20 | 80 ml | 600 µl |

2. Add 4 ml of cell lysis buffer to each labeled 50 ml polypropylene screw-cap tube.

3. Add plug(s) to tubes containing cell lysis buffer.

4. Incubate plugs in a **54ºC (+/- 1ºC)** shaker water bath (Lab-Line Instruments, Inc.) for 2 hours with constant agitation. When making duplicate plugs (two plugs of same isolate), both plugs may be lysed in the same tube.

**Note**: **All steps up to this point in the protocol should be done in sequence as outlined without delay.**

## 5.34 Washes

1. Wash plugs 2X with 15 ml of sterile Type 1 water (**preheated to 50º to 54ºC**) for 10 minutes at **50º to 54ºC** in a water bath with constant shaking. Wash 4X with 15 ml of TE, pH 8.0 (**preheated to 50º to 54ºC**) at **50º to 54ºC** for 15 minutes in a water bath with constant shaking.

2. After the last wash is completed, decant TE buffer and add 5 ml of fresh TE, pH 8.0, (room temperature) to each tube. Plugs slices (2-mm) may be restricted immediately with the appropriate enzyme or stored in TE at 4 – 6ºC.

**Note: *Salmonella* ser. Braenderup strain (H9812) is used as a reference standard. DNA of the H9812 strain must be digested with *Xba*I to give the appropriate band pattern. Follow instructions in the *E. coli* O157:H7 Standardized Laboratory protocol (*Section 5.11*) for making plugs and preparing restriction digests.**

## 5.35 Restriction Digestion of *L. monocytogenes* DNA in Agarose Plugs using *Asc*I

1. Dilute 10X Buffer 4 (New England BioLabs) 1:10 in sterile Type 1 water in a labeled Falcon 2057 tube according to Table 5.35a for the desired number of samples.

**Note: Wear gloves when handling 10X buffer. Keep buffer on ice.**

Table 5.35a

| Reagent | µl/Plug Slice | µl/13 Plug Slices |
|---|---|---|
| **Sterile Type 1 Water** | 135 µl | 1755 µl |
| **Buffer 4** | 15 µl | 195 µl |
| **Total Volume** | 150 µl | 1950 µl |

2. Add 150 µl diluted Buffer 4 to each labeled 1.5-ml microcentrifuge tube.

3. Carefully remove plug(s) from tube containing TE with wide end of the spatula. Cut off a 2.0 mm slice and add it to an appropriate labeled 1.5-ml microcentrifuge tube containing diluted Buffer 4. Be sure plug slice is submerged completely in the buffer.

**Notes: The shape and size of the plug slice that is cut will depend on the size of the teeth on the comb used for casting the gel. Gels wells that are cast with combs that have 10-mm-wide teeth will require a different size plug slice than those cast with combs with smaller teeth (5.5-mm). The number of slices that can be cut from the plugs will also depend on the skill and experience of the operator, integrity of the plug (i. e., whether it tore while doing the lysis and washing steps), and whether the slices are cut vertically of horizontally (5-mm x 10-mm plug). A small piece or the entire plug (10-mm x 5-mm) can be digested with restriction enzymes. Using only a small piece of the plug has some advantages: less restriction enzyme is used; the remainder of the plug is available to digest with enzyme s such as *Apa*1, *Sma*1, etc.**

**Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20ºC) at all times. Wear gloves.**

4. Replace rest of plug in original tube that contains TE Buffer.

5. Place tubes containing plug slices in Buffer 4 in a float and incubate in a 37ºC water bath for 10-15 minutes.

6. While plug slices are incubating in the Buffer 4, prepare the *Asc*1 enzyme mixture according to table 5.35b. The same Falcon 2057 tube that was used for Buffer 4 may be used.

Table 5.35b

| Reagent | µl/Plug Slice | µl/13 Plug Slices |
|---|---|---|
| **Sterile Type 1 Water** | 132.5 µl | 1722.5 µl |
| **Buffer 4** | 15 µl | 195 µl |
| **Enzyme (10 U/µl)** | 2.5 µl | 32.5 µl |
| **Total Volume** | 150 µl | 1950 µl |

7. At the end of the Buffer 4 incubation, remove Buffer 4 by inserting pipette fitted with 200-250 µl tip all the way to bottom of tube and aspirating the buffer. Be careful not to damage the plug slice with pipette tip and that plug slice is not discarded with the tip.

8. Add 150 µl of the *Asc*1 restriction enzyme mix to each tube. Close tube and mix by tapping gently on bench top; confirm that plug slice is submerged in the enzyme mix.

9. Place sample tubes in float and incubate in 37ºC water bath for at least 4 hr.

## 5.36 Restriction Digestion of *L. monocytogenes* DNA in Agarose Plugs using *Apa*I

1. Dilute 10X " A" Buffer (Roche Molecular Biochemicals) 1:10 in sterile Type 1 water in a labeled Falcon 2057 tube according to Table 5.36a for the number of samples desired.

**Note: Wear gloves when handling 10X buffer. Keep buffer on ice.**

Table 5.36a

| Reagent | µl/Plug Slice | µl/13 Plug Slices |
|---|---|---|
| **Sterile Type 1 Water** | 135 µl | 1755 µl |
| **"A" Buffer** | 15 µl | 195 µl |
| **Total Volume** | 150 µl | 1950 µl |

2. Add 150 µl diluted "A" Buffer to each labeled 1.5-ml microcentrifuge tube.

3. Carefully remove plug(s) from tube containing TE with wide end of the spatula. Cut off a 2.0-mm slice and add it to an appropriate labeled 1.5-ml microcentrifuge tube containing diluted "A" Buffer . Be sure plug slice is submerged completely in the buffer.

**Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20ºC) at all times. Wear gloves.**

4. Replace rest of plug in original tube that contains TE Buffer.

5. Place tubes containing plug slices in "A" Buffer in a float and incubate in a 37ºC water bath for 10 minutes.

6. While plug slices are incubating in the "A" Buffer , prepare the *Apa*1 enzyme mixture according to table 5.36b for the number of samples desired. The same Falcon 2057 tube that was used for "A" Buffer may be used.

Table 5.36b

| Reagent | µl/Plug Slice | µl/13 Plug Slices |
|---|---|---|
| **Sterile Type 1 Water** | 130 µl | 1690 µl |
| **"A" Buffer** | 15 µl | 195 µl |
| **Enzyme (40 U/µl)** | 5 µl | 65 µl |
| **Total Volume** | 150 µl | 1950 µl |

7. At the end of the "A" buffer incubation, remove "A" Buffer by inserting pipette fitted with 200-250 µl tip all the way to bottom of tube and aspirating the buffer. Be careful not to damage the plug slice with pipette tip and that plug slice is not discarded with the tip.

8. Add 150 µl of the *Apa*1 restriction enzyme mix to each tube. Close tube and mix by tapping gently on bench top; confirm that plug slice is submerged in the enzyme mix.

9. Place sample tubes in float and incubate in 30ºC water bath for at least 5 hours. Longer incubations may be required for some lots of *Apa*1. Sample tubes may be incubated overnight.

## 5.37 Casting PFGE Gel

1. Place a 15-well comb in 21 cm (8.5") wide x 14 cm (5.5") long gel form; use leveling platform to confirm that the gel is level. **Confirm that front of comb holder and teeth face the top of gel to allow maximum distance for DNA fragments to migrate, and that height of comb is 2-mm above the floor of the gel platform**. The same procedure may be used for 10-well comb in 14 cm (5.5") wide x 13 cm (5") long gel form.

**SAFETY WARNING**: Use insulated gloves when handling flasks after microwaving because they will be hot.

2. Weigh out the amount of SeaKem Gold (SKG) to make a 1% gel in 0.5X Tris-Borate EDTA Buffer.

3. Melt 1% SeaKem Gold (SKG) Agarose that was prepared with 0.5X Tris-Borate EDTA Buffer (TBE) as follows:

    a. Remove cap, cover top of flask loosely with clear plastic film, and microwave on medium power for 30 sec; mix gently and repeat for 10-20 sec intervals until completely melted.

    b. Recap flask and place melted agarose in 53º to 56ºC water bath for 5-6 minutes.

4. After agarose has cooled for 5-6 minutes, carefully pour agarose into gel casting unit. Remove any bubbles that form with a clean pipette tip.

5. Allow to solidify for at least 20 to 30 minutes before handling the gel or removing comb.

## 5.38 Preparation of Pulsed Field Electrophoresis System

1. Confirm that electrophoresis chamber is level; if necessary, adjust the leveling screws on the bottom of the unit. Put the black gel frame in the electrophoresis chamber; avoid touching the electrodes.

2. Add 2.2 L of 0.5X TBE; close the cover of the electrophoresis chamber.

3. Turn on cooling module and confirm that temperature setting is 14ºC.

4. Turn on power supply and pump; confirm that pump setting is 70 (the buffer flow at this setting should be approximately 1 liter/min) and that buffer is circulating through the tubing.

## 5.39 Loading Restricted plugs into the wells

1. Remove restricted plug slices from 30ºC or 37ºC water bath; allow to come to room temperature.

2. Remove enzyme/buffer mixture from plug slices with pipette and tip. Insert pipette fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to damage the plug slice with pipette tip and that plug slice is not discarded with the tip.

3. Add 200 µl 0.5X TBE to each plug slice.

4. Remove comb from gel.

5. Remove restricted **REFERENCE/STANDARD PLUG SLICES *Salmonella* ser. Braenderup (H9812)** with tapered end of spatula and place in wells 1, 5, and 10 for 10 well gel. For 15 well gels use three reference/standard plug slices (wells 1, 8, and 15). Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure there are no bubbles.

6. Place restricted sample plug slices in remaining wells; record order of loading.

7. Fill in wells of gel with melted 1.0% SKG agarose. Allow to harden for at least 5 minutes. Unscrew and remove end gates from gel casting unit; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on black casting platform and carefully place gel inside casting frame in electrophoresis chamber. Close cover.

**Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plugs in the wells so the lanes will be straight and the bands sharp. Plug slices can be loaded directly on the comb teeth before pouring the gel. For *Listeria,* the gel slices are loaded on the comb teeth inside the black support bar. Loading gel slices on the inside of the black support bar allows maximum distance for DNA fragments to migrate.**

**5.310 Electrophoresis Conditions**

1. Use the following electrophoresis conditions for *Asc*1or *Apa*1digested *L. monocytogenes* DNA plugs slices when using the Chef Mapper:

   Running buffer 0.5X TBE (Sigma); temperature = 14ºC
   Gel 1.0% SeaKem Gold Agarose prepared in 0.5X TBE

   Select Auto Algorithm on the Chef Mapper keypad
   Enter 30 kb for the Low MW; Enter 700 kb for the High MW
   Select default values by pressing **"Enter"**

   **For 14 cm (5.5") wide x 13 cm (5") long Gel change Run Time to 19 hours; press "Enter"**
   **For 21 cm (8.5") wide x 14 cm (5.5") long Gel change Run Time to 21 hours; press "Enter"**
   **Change Initial switch time to = 4.0 seconds**
   **Change Final switch time to = 40.0 seconds**
   **Press "Start Run"** (Note: Run-time is for 0.5X TBE, Sigma buffer)

2. When run is over, **TURN OFF 3 POWER SWITCHES**, open lid and remove gel.

**5.311 Ethidium bromide Gel Staining** (For detailed information see section 10)

1. Add 40 µl of 10 mg/ml ethidium bromide to 400 ml of reagent grade water in a container large enough to hold the gel to be stained. Place the gel in the solution.

2. Stain the gel for 20 to 30 minutes. Mild agitation will improve the diffusion of ethidium bromide into the gel.

3. Pour off the staining solution, and rinse once with water. Destain in water for 60 to 90 minutes. Changing the rinse water every 20-30 minutes will speed destaining.