

- 1. PURPOSE:** To describe the One-Day (24-26 h) standardized laboratory protocol for molecular subtyping of *Listeria monocytogenes* by Pulsed-field Gel Electrophoresis (PFGE).
- 2. SCOPE:** To provide the PulseNet participants with a standardized procedure for performing PFGE of *Listeria monocytogenes* thus ensuring inter-laboratory comparability of the generated results.
- 3. DEFINITIONS/TERMS:**
  - 3.1 PFGE: Pulsed-field Gel Electrophoresis
  - 3.2 DNA: Deoxyribonucleic acid
  - 3.3 CDC: Centers for Disease Control and Prevention
  - 3.4 CLRW: Clinical Laboratory Reagent Water

**4. RESPONSIBILITIES/PROCEDURE:**

**PREPARATION OF PFGE PLUGS FROM AGAR CULTURES**

**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. 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 all instructions carefully before starting protocol. Treat all plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs as contaminated materials and dispose of, or disinfect according to the guidelines of your institution. Disinfect reusable plug molds before they are washed; the disposable plug molds, including the tape and the tab that is used to push the plugs out of the wells, are also contaminated and should be disinfected with 1% Lysol/Amphyll or 90% ethanol for at least 30 minutes if they will be washed and reused.

**Day 0**

Streak an isolated colony from test cultures onto Brain Heart Infusion Agar (BHIA) plates (or comparable non-selective media) for confluent growth. It is recommended that a storage vial of each culture be created. To do this, stab small screw cap tubes of TSA, HIA, or similar medium with the same inoculating loop used to streak the plate. This will ensure that the same colony can be retested if necessary. Incubate cultures at 37°C for 14-18 h.

**Day 1**

- 1. Turn on shaker water bath or incubator (54-55°C), stationary water baths (55-60°C) and spectrophotometer** (or equivalent instrument such as the Dade Microscan Turbidity meter or bioMérieux Vitek colorimeter).
- Prepare **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)**<sup>1</sup> as follows:
  - 10 ml of 1 M Tris, pH 8.0
  - 2 ml of 0.5 M EDTA, pH 8.0
  - Dilute to 1000 ml with sterile Ultrapure water (Clinical Laboratory Reagent Water (CLRW))

**Note:** The TE Buffer is used to make the plug agarose, suspend the cells, and to wash lysed PFGE plugs.

- Prepare Lysozyme stock solution as follows:

<sup>1</sup>Additional information is found on page 12 of this document.

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- a. For 20mg/ml stock, weigh out 100 mg Lysozyme (Sigma L7651 or L6875). Keep the container of Lysozyme on ice.
  - b. Add 5 mL TE buffer, swirl to mix,
  - c. Aliquot 250  $\mu$ L amounts into sterile eppendorf tubes and freeze for future use.
  - d. Or for 10mg/ml stock, weight out 50 mg Lysozyme (Sigma L1667). Keep the container of Lysozyme on ice.
  - e. Add 5 mL TE buffer, swirl to mix.
  - f. Aliquot 250  $\mu$ L amounts into sterile eppendorf tubes and freeze for future use.
4. Prepare 1% SeaKem Gold agarose + 0.5% Sodium Dodecyl Sulfate (SDS) in **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** for PFGE plugs as follows:
- a. Place 10% SDS stock solution (GibcoBRL #15553-035) into 55- 60°C water bath to warm.
  - b. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.
  - c. Add 47.5 ml (or 23.75 ml) TE Buffer; swirl gently to disperse agarose.
  - d. Loosen or remove cap and cover loosely with clear film, and microwave for 30 sec; mix gently and repeat for 10 sec intervals until agarose is completely dissolved.
  - e. Add 2.5 mL (or 1.25 mL) of warm 10% SDS stock solution, swirl to mix.
  - f. Recap flask and return to 55- 60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

**SAFETY WARNING:** Use heat-resistant gloves when handling hot flasks after microwaving.

**Note:** SeaKem Gold agarose works well for making PFGE plugs because it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used, and will have to be determined empirically in each laboratory.

5. Label small transparent tubes (12 mm x 75 mm Falcon 2054 tubes or equivalent) with culture numbers.
6. Transfer  $\approx$ 2 ml of **TE Buffer** to small labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile TE to remove some of the growth from the agar plate; suspend cells in TE by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

**Note:** The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process at one time.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile TE or by adding additional cells.
  - a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of **1.00**
  - b. Dade Microscan Turbidity Meter: **0.40 - 0.45** (measured in Falcon 2054 tubes)  
**0.58 - 0.63** (measured in Falcon 2057 tubes)
  - c. bioMérieux Vitek colorimeter:  $\approx$ **17-18%** transmittance (measured in Falcon 2054 tubes)

**Note:** Cell suspensions need to be at room temperature when concentration is checked. The values in Steps 7a, 7b and 7c give satisfactory results at CDC; if different instruments or tubes are used, each laboratory may need to establish the concentration needed for satisfactory results.

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### CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

**Note:** Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low-medium power for 10 -15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted. This agarose melts rapidly!

**Note:** Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure water (CLRW). For best results, aliquot 300-500 µl into small tubes and store in a freezer (-20°C) until ready to use. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. If the Proteinase K stock solution was prepared from powder, discard any thawed solution at the end of the work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5 ml microcentrifuge tubes.
2. Add 20 µL thawed Lysozyme stock solution (20 mg/mL or 10 mg/mL<sup>2</sup>) to each tube and mix gently. Place tubes into a 55-60°C water bath for 10-20 minutes. Discard unused thawed Lysozyme solution.
3. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl is needed for 10 cell suspensions.)
4. Add 400 µl (0.4 ml) melted 1% SeaKem Gold agarose to 400 µl cell suspension and mix by gently pipetting mixture up and down a few times. **Over-pipeting can cause DNA shearing.** Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60°C).
5. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4°C) for 5 minutes.

**Note:** If disposable plug molds are used for making plugs with 1% SeaKem Gold agarose, use 200 µl cell suspension, 10 µl of Lysozyme (20 mg/ml), 10 µl of Proteinase K (20 mg/ml stock), and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

**Note:** The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches.

### LYSIS OF CELLS IN AGAROSE PLUGS

**Note:** Two plugs (reusable plug molds) or up to four plugs (disposable plug molds) of the same strain can be lysed in the same 50 ml tube.

1. Label 50 ml polypropylene screw-cap or 50 ml Oak Ridge tubes with culture numbers.

<sup>2</sup> The same volume is added of either stock solution because L1667 has approximately twice the activity of L6875 or L7651, according to Sigma.

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2. Prepare **Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl)** as follows:  
25 ml of 1 M Tris, pH 8.0  
50 ml of 0.5 M EDTA, pH 8.0  
50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)<sup>3</sup>  
Dilute to 500 ml with sterile Ultrapure water (CLRW)
3. Calculate the total volume of **Cell Lysis/Proteinase K Buffer** needed as follows:
  - a. 5 ml **Cell Lysis Buffer** (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).
  - b. 25 µl **Proteinase K** stock solution (20 mg/ml) is needed per tube of the cell lysis buffer (e. g., 25 µl x 10 tubes = 250 µl).
  - c. Prepare the master mix by measuring the correct volume of Cell Lysis Buffer and Proteinase K into appropriate size test tube or flask and mix well.

**Note:** The final concentration of Proteinase K in the lysis buffer is **0.1 mg/ml** and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.
5. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Open reusable plug mold and transfer plugs from mold with a 6 mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from the bottom of mold and push out plug(s) into appropriately labeled tube. **Be sure plugs are under buffer and not on side of tube.**

**Note:** The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. **Remove tape from reusable mold.** Place both sections of the plug mold, spatulas, and scalpel in 90% ethanol, 1% Lysol/Amphyll or other suitable disinfectant. **Soak them for 15 minutes before washing them.** Discard disposable plug molds.
7. Place tubes in rack and incubate in a 54-55°C shaker water bath or incubator for 2 h with **constant and vigorous agitation** (150-175 rpm). If lysing in water bath, be sure water level is **above** level of lysis buffer in tubes.
8. Pre-heat enough sterile Ultrapure water (CLRW) to 54-55°C so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

**WASHING OF AGAROSE PLUGS AFTER CELL LYSIS**

**Note:** Most laboratories will find that their plugs are sufficiently stable to perform the following washing steps at 54-55°C. However, if you notice that your plugs are nicked along the edges or breaking it will be necessary for your laboratory to lower the water bath or incubator to 50°C for the following washing steps.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

<sup>3</sup>The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve. See page 12 of this document.

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**Note:** It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure water (CLRW) that has been pre-heated to 54-55°C to each tube and shake the tubes in a 54-55°C water bath or incubator for 10-15 min.
3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.
  - a. Pre-heat enough sterile **TE Buffer** (10 mM Tris:1 mM EDTA, pH 8.0) in a 54-55°C water bath so that plugs can be washed four times with 10-15 ml TE (400-600 ml for 10 tubes) after beginning last water wash.
4. Pour off water, add 10-15 ml pre-heated (54-55°C) sterile TE Buffer, and shake the tubes in 54-55°C water bath or incubator for 10-15 min.
5. Pour off TE and repeat wash step with pre-heated TE three more times.
6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4°C until needed. Plugs can be transferred to smaller tubes for storage.

**Note:** If restriction digestion is to be done the same day, complete Steps 1-3 of next section (**RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *AscI* or *ApaI***) during last TE wash step for optimal use of time.

**RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS**

**Note:** A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme *AscI* because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. *ApaI* is recommended as the secondary enzyme for analysis of *Listeria monocytogenes* isolates. The use of a secondary enzyme is useful in situations where the PFGE patterns obtained with the primary enzyme are also indistinguishable.

1. Label 1.5 ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812<sup>4</sup> standards.
  - a. **Pre-Restriction Incubation Step (highly recommended):** Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche Applied Science or equivalent) 1:10 with sterile Ultrapure water (CLRW) according to the following table:

**Note:** Use the appropriate buffer supplied by the enzyme manufacturer. For NEB enzymes, the corresponding buffer for *AscI* and *ApaI* is NEBuffer 4. For Roche the corresponding buffer for *ApaI* is buffer A. Roche does not sell *AscI*.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
<b>Sterile Clinical Laboratory Reagent Water (CLRW)</b>	180 µl	1800 µl	2700 µl
<b>10X Restriction Buffer</b>	20 µl	200 µl	300 µl

<sup>4</sup>Directions for making and testing PFGE plugs of *Salmonella* ser. Braenderup H9812 are in PNL05.

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<b>Total Volume</b>	200 µl	2000 µl	3000 µl
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- b. Add 200 µl diluted restriction buffer (1X) to labeled 1.5 ml microcentrifuge tubes.
- c. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
- d. Cut a 2.0 to 2.5 mm-wide slice from each test sample and the appropriate number of *S. ser. Braenderup H9812* standards with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted restriction buffer. **Be sure plug slice is under buffer.** Replace rest of plug in the original tube that contains 5 ml TE buffer and store at 4°C.

**Note:** PulseNet recommends that the combs with larger teeth (10 mm-wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5 mm). Using combs with smaller teeth is not advised. The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

- e. Incubate sample and control plug slices in water bath or incubator for 5-10 min or at room temperature for 10-15 min.
    - i. Incubate samples to be restricted with *AscI* and *XbaI* at 37°C.
    - ii. Incubate samples to be restricted with *ApaI* at 30°C (Roche) or 25°C (New England Biolabs).
  - f. After incubation, remove buffer from plug slice using a pipet 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 pipet tip and that plug slice is not discarded with pipet tip.
2. Prepare the restriction enzyme master mix according to the following table. May mix in the same tube that was used for the diluted restriction buffer:

**Note:** Enzymes may be purchased in several different stock concentrations. The calculations outlined here are based on using an enzyme at a concentration of 10 or 50 U/µl. If the enzyme used is of a different concentration, make necessary adjustments to the volume of enzyme and water to achieve a final concentration of **25 U/ sample**.

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

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
<b>Sterile Clinical Laboratory Reagent Water (CLRW)</b>	175.5 µl	1755 µl	2632.5 µl
<b>10X Corresponding Restriction Buffer</b>	20 µl	200 µl	300 µl
<b>BSA (10mg/ml)</b>	2 µl	20 µl	30 µl
<b><i>AscI</i> (10 U/µl)</b>	2.5 µl	25 µl	37.5 µl
<b>Total Volume</b>	200 µl	2000 µl	3000 µl

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
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<b>Sterile Clinical Laboratory Reagent Water (CLRW)</b>	177.5 µl	1775 µl	2662.5 µl
<b>10X Corresponding Restriction Buffer</b>	20 µl	200 µl	300 µl
<b>BSA (10mg/ml)</b>	2 µl	20 µl	30 µl
<b><i>ApaI</i> (50 U/µl)</b>	0.5 µl	5 µl	7.5 µl
<b>Total Volume</b>	200 µl	2000 µl	3000 µl

**Note: Addition of Bovine Serum Albumin (BSA; highly recommended):** Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures while others do not. PulseNet Central recommends adding BSA to all enzyme restriction mixtures to minimize the incidence of incomplete restriction.

- Add 200 µl restriction enzyme master mix to each tube. Close tube and mix by tapping gently; **be sure plug slices are under enzyme mixture.**
- Incubate sample and standard (control) plug slices for 2 hours in a water bath at the appropriate temperature for the enzyme.
  - Incubate samples restricted with *ApaI* at 25°C.
  - Incubate samples restricted with *AscI* and *XbaI* at 37°C.
- If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (**CASTING AGAROSE GEL**) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

**CASTING AGAROSE GEL**

**A. Loading Restricted Plug Slices on the Comb:**

- Confirm that water bath is equilibrated to 55-60°C.
- Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

**5X TBE:**

Reagent	Volume in milliliters (ml)	
<b>5X TBE</b>	200	220
<b>Clinical Laboratory Reagent Water (CLRW)</b>	1800	1980
<b>Total Volume of 0.5X TBE</b>	2000	2200

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**10X TBE:**

Reagent	Volume in milliliters (ml)	
<b>10X TBE</b>	100	110
<b>Clinical Laboratory Reagent Water (CLRW)</b>	1900	2090
<b>Total Volume of 0.5X TBE</b>	2000	2200

3. Make 1% SeaKem Gold (SKG) agarose in 0.5X TBE as follows:
  - a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
  - b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.
    - i. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14 cm wide gel form (10 wells)
    - ii. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21 cm wide gel form (15 wells)
  - c. Loosen cap and microwave for 60 sec; mix gently and repeat for 15 sec intervals until agarose is completely dissolved.
  - d. Recap flask and return to 55-60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

**SAFETY WARNING:** Use heat-resistant gloves when handling hot flasks after microwaving.

**Note:** Agarose LF™ (Amresco, X174) is the only acceptable alternative to SeaKem Gold, at this time. The time and temperature needed to completely dissolve the agarose is dependent on the specifications of the microwave used and will have to be determined empirically in each laboratory. Similarly, the optimal running time for each agarose will have to be determined empirically in each laboratory.

4. A small volume (2-5 ml) of melted and cooled (50-60°C) 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several times.

**Note:** Place the gel form on a leveling table and adjust until perfectly leveled. Place the comb holder so the front part (side with small metal screws) and teeth face the bottom of gel frame and the comb teeth touch the gel platform.

5. Remove restricted plug slices from the water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.
6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:
  - a. Load *S. ser.* Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10-well gel) or on teeth 1, 5, 10, 15 (15-well gel).
  - b. Load samples on remaining teeth and note locations.
7. Remove excess buffer with tissue or kimwipe. Allow plug slices to air dry on the comb for 5-10 minutes or seal them to the comb with 1% SKG agarose (55-60°C).
8. Position comb in leveled gel form and confirm that the plug slices are correctly aligned on the bottom of the comb teeth, and that the lower edge of the plug slice is flush against the black platform.
9. Carefully pour the agarose (cooled to 55-60°C) into the gel form and remove any bubbles or debris.

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10. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.
11. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting of ~70) and cooling module (14°C) approximately 30 minutes before gel is to be run.
12. Remove comb after gel solidifies, about 30-45 minutes.
13. Fill in wells of gel with melted and cooled (55-60°C) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

**B. Loading Restricted Plug Slices into the Wells:**

1. Follow steps 1-4 in Option A on pages 7 and 8 (**Loading Restricted Plug Slices on the Comb**).

**Note:** Place the gel form on a leveling table and adjust until perfectly leveled before pouring gel. Position the comb holder so that front part (side with small metal screws) and teeth face the bottom of gel and the **bottom edge of the comb is 2 mm above the surface of the gel platform.**

2. Cool melted SKG agarose in 55-60°C water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.
3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)
4. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (set at ~70) and cooling module (14°C) approximately 30 min before gel is to be run.
5. Remove restricted plug slices from 37°C water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.
6. Remove comb after gel solidifies for at least 30 minutes.
7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.
  - a. Load *S. ser. Braenderup H9812* standards in wells (lanes) 1, 5, 10 (10 well gel) or in wells 1, 5, 10, 15 (15 well gel).
  - b. Load samples in remaining wells.

**Note:** Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

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8. Fill in wells of gel with melted 1% SKG agarose (equilibrated to 55-60°C). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue or kimwipe. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

**ELECTROPHORESIS CONDITIONS**

1. Select following conditions for *Listeria monocytogenes* strains restricted with *AscI* or *ApalI*:

- a. Select following conditions on **CHEF Mapper**

Auto Algorithm

49 kb - low MW

450 kb - high MW

Select default values except where noted by pressing "enter."

Initial switch time = 4.0 s

Final switch time = 40.0 s

**Change run time to 18 - 19 h** (See note below)

- b. Select following conditions on **CHEF-DR III**

Initial switch time: 4.0 s

Final switch time: 40.0 s

Voltage: 6 V

Included Angle: 120°

Run time: 18-19 h (See note below)

- c. Select following conditions on **CHEF-DR II**

Initial A time: 4.0 s

Final A time: 40.0 s

Start ratio: 1.0 (if applicable)

Voltage: 200 V

Run time: 19-20 h (See note below)

**Note:** The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. **Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S. ser. Braenderup H9812* standard migrates 1.0 - 1.5 cm from the bottom of the gel.**

**Note:** Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-150 mAmps. A reading outside of this range may indicate that the 0.5X TBE buffer was prepared improperly and the buffer should be remade.

**Day 2**

**STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL**

**Note:** The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the "Alternate DNA Stains-Results and Recommendations" posting within the PulseNet Documents forum on the SharePoint site for additional information.

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**STANDARD OPERATING PROCEDURE FOR PULSENET PFGE  
OF *LISTERIA MONOCYTOGENES***

**CODE: PNL04**

Effective Date:

03	24	2013
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1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide by diluting 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml CLRW. This volume is for a staining box that is approximately 14 cm x 24 cm; a larger container may require a larger amount of staining solution). Stain gel for 20 - 30 min in covered container.

**Note:** Ethidium bromide is toxic and a mutagen. Stock solutions of 10 mg/ml Ethidium Bromide (EtBr) in water are available from several commercial companies (Amresco X328; Bio-Rad, 161-0433; Sigma, E-1510). The diluted solution can be kept in dark bottle and reused 6-8 times before discarding according to your institution's guidelines for hazardous waste. CDC does not recommend disposing of EtBr down the drain. Aqueous solutions containing EtBr can be filtered through charcoal or degraded using activated carbon destaining or "tea" bags from Amresco (E732-25 Destaining Bags) or other companies, which effectively and safely remove EtBr from solutions and gels. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. If you have further questions about EtBr please refer to the **Material Safety Data Sheets (MSDS)** provided by the vendor or manufacturer.

**Note:** Currently, the only acceptable alternative stain options are GelRed™ (Biotium, 31010), SYBR® Safe (Invitrogen, S-33102) and SYBR® Gold (Invitrogen, S-11494). Labs are strongly encouraged to follow manufacturer's instructions and test stains in their labs before adopting them for routine use. If one of the alternative stains is used, the destaining steps should be omitted.

2. Destain gel in approximately 500 ml CLRW for 60 - 90 min, changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.
3. Follow directions given with the imaging equipment to save gel image as a \*.1sc file; convert this file to \*.tif file for analysis with the BioNumerics software program. The gel image should fill the entire window of the imaging equipment (computer) screen (without cutting off wells or lower bands). Ensure that the image is in focus and that there is little to no saturation (over-exposure) in the bands (signified by red pixilation in the QuantityOne or ImageLab software). Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.
4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L CLRW or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min **before** draining water from chamber and hoses.
5. If the lowest band in the H9812 standard does not migrate within 1-1.5 cm of the bottom of the gel, the proper run time will need to be determined empirically for the conditions in each laboratory.

**Note:** The following options are available if PFGE results do not have to be available within 24-28 hours:

- Plugs can be lysed for longer periods of time (3-16 hours).
- The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

Use of trade names and commercial sources is for identification purposes only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

**NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS**

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Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

Any questions regarding the CLIA requirements for a procedure manual, quality control, quality assurance, etc., should be directed to the agency or accreditation organization responsible for performing your laboratory's CLIA inspection. In addition, some states and accreditation organizations may have more stringent requirements that will need to be addressed.

**Formulas of Selected Reagents used in PulseNet Standardized Laboratory Protocol for PFGE**

**Tris:EDTA Buffer, pH 8.0 (TE, 10 mM Tris:1 mM EDTA, pH 8.0)**

10 ml of 1 M Tris, pH 8.0  
2 ml of 0.5 M EDTA, pH 8.0  
Dilute to 1000 ml with sterile Ultrapure Water (CLRW)

**Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosine + 0.1 mg/ml Proteinase K)**

25 ml (50 ml) of 1 M Tris, pH 8.0  
50 ml (100 ml) of 0.5 M EDTA, pH 8.0  
50 ml (100 ml) 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl)

**or**

5 g (10 g) of N-Lauroylsarcosine, Sodium salt (Sarcosyl)<sup>5</sup>  
Dilute to 500 ml (1000 ml) with Sterile Ultrapure Water (CLRW)

Add **25 µl** Proteinase K stock solution (20 mg/ml) per 5 ml of cell lysis buffer **just before use** for a final concentration in the lysis buffer of 0.1 mg/ml Proteinase K.

**5. FLOW CHART:**

**6. BIBLIOGRAPHY:**

**7. CONTACTS:**

**8. AMENDMENTS:**

8.1 The above protocol was revised and distributed on October 30, 2008, modifications as follows:

- Reduction of cell suspension concentration
- Solubilization of the Lysozyme in TE rather than water
- Volumes used for plug preparation
- Incubation of the cell suspensions with the Lysozyme for 10-20 minutes in a 56°C water bath rather than 37°C
- Restriction enzyme units used for *ApaI* (50 U/sample) and *AscI* (40 U/ sample) for two to three hours

8.2 2011-07 A note was added to clarify which buffers to use during the restriction digestion "step 2" under the enzyme mixture charts.

<sup>5</sup>If Sarcosyl powder is added directly to the other components of this reagent, warm the solution to 50-60°C for 30-60 minutes, or leave at room temperature for ≈2 hours to completely dissolve the Sarcosyl; adjust to the final volume with sterile Sterile Clinical Laboratory Reagent Water.

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8.3 2013-03 changes:

- Corrected formula for TE buffer. TE used at CDC is 10 for Tris and 1mM for EDTA.
- Recommended disinfectant changed from 10% bleach to 1% Lysol/Amphyll or 90% ethanol.
- Corrected calculations for plug agarose.
- New options for Lysozyme were added because Sigma L7651 was discontinued.
- A note was added to provide guidance when working with large numbers of isolates (>10).
- Volume of TE needed for washing plugs was corrected from 300 – 350 ml to 400 – 600 ml.
- Use of pre-restriction step was changed from optional to highly recommended.
- A statement was added to clarify that using combs with smaller teeth (5.5 mm) was not advised.
- Units of enzyme for *AscI* and *ApaI* was reduced from 40 and 50 units, respectively, to 25 units for each. Incubation time was changed to 2 hours, instead of a range of 2 – 3 hours.
- A statement was included to allow the use of an alternative agarose for casting the running gel, along with recommendations strongly urging each lab to optimize the run time. Internal and external validation showed that run times could be affected by agarose type, but no trends were noted so a blanket recommendation on run times cannot be made. Additional agarose alternatives may be tested and deemed acceptable at a later date.
- A statement was included to allow the use of alternative DNA stains that are equivalent to EtBr. Labs are strongly urged to follow manufacturer's instructions as well as test stains in their own labs to gain experience using alternative agarose stains. Additional stain alternatives may be tested and deemed acceptable at a later date.

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