

Standard Operating Procedure for PulseNet PFGE of *Listeria monocytogenes*

Purpose

To describe the One-Day (24-26 hour) Standardized Laboratory Protocol for Molecular Subtyping of *Listeria monocytogenes* by Pulsed-field Gel Electrophoresis (PFGE).

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.

Definitions and Terms

1. PFGE: Pulsed-field Gel Electrophoresis
2. DNA: Deoxyribonucleic acid
3. CDC: Centers for Disease Control and Prevention
4. CLRW: Clinical Laboratory Reagent Water
5. EDTA: Ethylenediaminetetraacetic acid
6. TE: Tris-EDTA
7. TBE: Tris borate-EDTA
8. TSA: Trypticase Soy Agar
9. HIA: Heart Infusion Agar

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. It is recommended to plate cultures, prepare cell suspensions, and cast plugs in a Class II Biosafety Cabinet (BSC), if available. 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 your institutional guidelines.

Day 0

Plating for confluent growth

1. 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.
2. Incubate cultures at 37°C for 14-18 hours.

Day 1

Preparing Cell Suspensions

1. Turn on shaker water bath or incubator (54-55°C), stationary water baths (55-60°C) and if applicable, the spectrophotometer used for measuring optical densities of cell suspensions during plug preparation.
2. Prepare TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) as follows:
 - 2.1. 10 ml of 1 M Tris, pH 8.0
 - 2.2. 2 ml of 0.5 M EDTA, pH 8.0
 - 2.3. Dilute to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent Water (CLRW)

Alternatively, 0.01M TE Buffer may be purchased from a commercial vendor. The TE Buffer is used to make the plug agarose and also to wash lysed PFGE plugs.

3. Prepare Lysozyme stock solution as follows:
 - 3.1. For 20 mg/ml stock, weigh out 100 mg Lysozyme (Sigma L4919 or L6876). Keep the container of Lysozyme on ice.
 - 3.2. Add 5 ml TE buffer, swirl to mix,
 - 3.3. Aliquot 250 µl amounts into sterile eppendorf tubes and freeze for future use.
 - 3.4. Or for 10 mg/ml stock, weight out 50 mg Lysozyme (Sigma L1667). Keep the container of Lysozyme on ice.
 - 3.5. Add 5 mL TE buffer, swirl to mix.
 - 3.6. Aliquot 250 µL 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 for PFGE plugs as follows:
 - 4.1. Place 10% SDS stock solution (GibcoBRL/ThermoFisher BRL #15553-035) into 55-60°C water bath to warm.
 - 4.2. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.
 - 4.3. Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.
 - 4.4. Loosen cap or cover loosely with clear film and microwave for 30 seconds; mix gently and repeat for 10 seconds intervals until agarose is completely dissolved.
 - 4.5. Add 2.5 ml (or 1.25 ml) of warm 10% SDS stock solution and swirl to mix.
 - 4.6. 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.

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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 (12mm x 75mm Falcon 2054 tubes or equivalent) with culture numbers.

6. Transfer 2 ml of **TE buffer** to small labeled tubes. 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.
7. 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. Place suspensions on ice if you have more than 6 cultures to process at one time.
8. Adjust concentration of cell suspensions to one of values given below by diluting with sterile TE or by adding additional cells using a swab to remove more growth from the agar plate:
 - 8.1. Spectrophotometer: 610 nm wavelength, **Optical Density of 1.00**
 - 8.2. Microscan Turbidity Meter (Beckman Coulter):
 - **0.40-0.45** (measured in Falcon 2054 tubes)
 - **0.58-0.63** (measured in Falcon 2057 tubes)
 - 8.3. bioMérieux Vitek colorimeter: **17-18% transmittance** (measured in Falcon 2054 tubes)

The values in step 8.1 – 8.3 give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

Casting Plugs

The preparation of cell suspensions and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis and solidification of agarose in pipette tips/microcentrifuge tubes. If large numbers of samples are being prepared, it is recommended to process them in batches of ~10 samples at a time. Once the first batch of isolates are in the cell lysis incubation step, then the cell suspensions can be prepared for the next group of samples, and so on. All batches can be lysed and washed together since additional lysis time will not affect the initial batches. Unused plug agarose can be kept at room temperature and reused 1 or 2 times. Microwave on low-medium power for 10-15 sec and mix; repeat for 5-10 sec intervals until agarose is completely melted.

1. 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.
2. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5-ml microcentrifuge tubes.
3. Add 20 µL thawed Lysozyme stock solution (20 mg/mL or 10 mg/mL¹) to each tube and mix gently. Place tubes into a 55-60°C water bath for 10-20 minutes. Discard unused thawed Lysozyme solution.
4. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip.

Proteinase K solutions (20 mg/ml) are available commercially. Alternatively, a stock solution of Proteinase K can be prepared from powder in sterile Ultrapure water (CLRW; see PNL02). 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 workday. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

5. Add 400 µl melted 1% SeaKem Gold agarose to 400 µl cell suspension and mix by gently pipetting mixture up and down two or three times. **Over-pipeting can cause DNA shearing.** Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60°C).
6. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold without introducing bubbles. Two plugs of each sample can be made from these amounts of cell suspension and agarose and are useful if repeat testing is

¹ The same volume is added of either stock solution because L1667 has approximately twice the activity of L6876 or L7651, according to Sigma.

required. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4°C) for 5 minutes.

If disposable plug molds are used combine 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 smaller volumes.

Lysis of Cells in Agarose Plugs

Two plugs (reusable molds) or up to 4 plugs (disposable molds) of the same strain can be lysed in the same 50ml tube.

1. Label 50ml polypropylene screw-cap tubes with culture numbers.
2. Calculate the total volume of **Cell Lysis Buffer/Proteinase K** master mix needed as follows:
 - 2.1. 5 ml stock **Cell Lysis Buffer** (CLB; 50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl per tube as prepared in "Formulas of PFGE Reagents" section)
 - e. g., 5 ml x 10 tubes = 50 ml
 - 2.2. 25 µl **Proteinase K** stock solution (20 mg/ml) per tube of the CLB
 - e. g., 25 µl x 10 tubes = 250 µl
 - 2.3. Prepare the master mix by measuring the correct volume of CLB and Proteinase K into appropriate size test tube or flask and mix well.
3. Aliquot 5 ml of **Cell Lysis Buffer/Proteinase K** master mix to each labeled 50 ml tube.
4. Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument (optional). Open reusable plug mold and transfer plugs from mold with a 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.**

The excess agarose, plug mold, spatula, etc. are contaminated. Dispose of or disinfect them appropriately.

5. Remove tape from reusable mold. Place both sections of the plug mold, spatulas, and scalpel 90% ethanol, 70% isopropanol, 1% Lysol or other suitable disinfectant. **Soak them for at least 30 minutes before washing them.** Discard disposable plug molds appropriately.
6. Place tubes in a non-Styrofoam rack and incubate in a 54-55°C shaker water bath or incubator for 2 h with **constant and vigorous agitation** (175-200 rpm).
7. If lysing in water bath, be sure water level in water bath is **above** level of lysis buffer in tubes.
8. Pre-heat enough sterile CLRW and TE Buffer to 54-55°C so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes) and washed four times with 10-15 ml TE (400-600 ml for 10 tubes).

Washing of Agarose Plugs After Cell Lysis

Plug washing can be started on Day 1 and finished the morning of Day 2 after overnight refrigeration of the plugs in TE (after completion of two water and two TE washes).

Most laboratories will find that plugs are sufficiently stable to perform the following washing steps at 54-55°C. However, if plugs are nicked along the edges or breaking, lower the temperature to 50°C for the following washing steps. Washes at lower temperatures should be increased to up to 30 min.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into container of suitable disinfectant; plugs can be held in tubes with a screened cap (Bio-Rad) or spatula.

- Add 10-15 ml of pre-heated (54-55°C), sterile CLRW to each tube and shake the tubes in a 54-55°C water bath or incubator for 10-15 min.
- Pour water down drain or treat subsequent washes according to laboratory's waste disposal guidelines and repeat wash step with pre-heated water one more time.
- 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 minutes.
- Pour off TE and repeat wash step with pre-heated TE three more times.
- Decant last wash and add 5-10 ml sterile TE. Continue with "Restriction Digestion of DNA in Agarose Plugs" section or store plugs in TE Buffer at 4°C until needed. Plugs can be transferred to smaller tubes for long-term storage.

If restriction digestion is to be done the same day, complete Steps 1-3 of next section (Restriction Digestion of DNA in Agarose Plugs) during last TE wash step for optimal use of time.

Restriction Digestion of DNA in Agarose Plugs

*A small slice of the plug (not the entire plug) should be digested with the primary restriction enzyme *Ascl* because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. *Apal* 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.*

- Label 1.5-ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812² standards.

*The number of the *Salmonella* serotype Braenderup H9812 standards is dependent on the number of samples being run on the gel. Standards should be placed on the ends and between samples as needed; no more than 4 samples should be placed between the standard lanes.*

- 1.1. Pre-Restriction Incubation Step (highly recommended):** Prepare a master mix by diluting the appropriate 10X restriction buffer (Roche /Sigma, New England Biolabs or equivalent) 1:10 with sterile CLRW according to the following table:

Reagent	µl/Plug Slice	µl/7Plug Slices	µl/11 Plug Slices
CLRW	180 µl	1260 µl	1980 µl
10X Restriction Buffer	20 µl	140 µl	220 µl
Total Volume	200 µl	1400 µl	2200 µl

*The appropriate restriction buffer varies between vendors and may differ between enzymes from the same vendor. **Always** use the restriction buffer recommended by the vendor for the particular restriction enzyme.*

- Add 200 µl diluted restriction buffer (1X) to labeled 1.5-ml microcentrifuge tubes.
- Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
- Cut a 2.0 to 2.5mm wide slice from each test samples 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 (short-term storage) that contains at least 5 ml TE buffer or smaller tube containing TE buffer (long-term storage). Plugs should be stored at 4°C.

² Directions for making and testing PFGE plugs of *Salmonella ser. Braenderup H9812* are in PNL05

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 (<10 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).

5. Incubate sample and control plug slices in water bath for 5-10 minutes or at room temp for 10-15 minutes.
 - 5.1. Incubate samples to be digested with *Ascl* and *XbaI* at 37°C
 - 5.2. Incubate samples to be digested with *Apal* at 30°C (Roche/Sigma) or 25°C (New England BioLabs)
6. 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.
7. Prepare the restriction enzyme master mix according to the following table. Keep vials of restriction enzyme on ice or in an insulated storage box (-20°C) at all times.

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.

Reagent	µl/Plug Slice	µl/7 Plug Slices	µl/11 Plug Slices
CLRW	176.5 µl	1235.5 µl	1941.5 µl
10X Restriction Buffer	20 µl	140 µl	220 µl
BSA (20mg/ml)	1 µl	7 µl	11 µl
<i>Ascl</i> (10U/ µl)	2.5 µl	17.5 µl	27.5 µl
Total Volume	200 µl	1400 µl	2200 µl

Reagent	µl/Plug Slice	µl/7 Plug Slices	µl/11 Plug Slices
CLRW	178.5 µl	1249.5 µl	1963.5 µl
10X Restriction Buffer	20 µl	140 µl	220 µl
BSA (20mg/ml)	1 µl	7 µl	11 µl
<i>Apal</i> (50U/ µl)	0.5 µl	3.5 µl	5.5 µl
Total Volume	200 µl	1400 µl	2200 µl

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. BSA may be purchased in different stock concentrations. The calculations above are based on using BSA at a concentration of 20 mg/ml. If a different concentration of BSA is used, make necessary adjustments to the volume of BSA and water to achieve a final concentration of 0.1 mg/ml.*

8. 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.**
9. Incubate sample and standard (control) plug slices for 2 hours in a water bath at the appropriate temperature for the enzyme.
 - 9.1. Incubate samples restricted with *Apal* at 25°C.
 - 9.2. Incubate samples restricted with *Ascl* and *XbaI* at 37°C.

10. If plug slices will be loaded into the wells the same day, continue with the next section (**CASTING AN AGAROSE GEL**) approximately 1 hour before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

Casting an Agarose Gel

1. Confirm that water bath is equilibrated to 55-60°C.
2. 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)			
	(DR-II) Running buffer + 10 well gel prep	(DR-II) Running buffer + 15 well gel prep	(CHEF Mapper/DRIII) Running buffer + 10 well gel prep	(CHEF Mapper/DRIII) Running buffer + 15 well gel prep
5X TBE	210	215	230	235
CLRW	1890	1935	2070	2115
Total Volume of 0.5X TBE	2100	2150	2300	2350

10X TBE:

Reagent	Volume in milliliters (ml)			
	(DR-II) Running buffer + 10 well gel prep	(DR-II) Running buffer + 15 well gel prep	(CHEF Mapper/DRIII) Running buffer + 10 well gel prep	(CHEF Mapper/DRIII) Running buffer + 15 well gel prep
10X TBE	105	107.5	115	117.5
CLRW	1995	2042.5	2185	2232.5
Total Volume of 0.5X TBE	2100	2150	2300	2350

3. Make 1% SeaKem Gold (SKG; the only acceptable agarose to be used for PulseNet PFGE) agarose in 0.5X TBE as follows:
 - 3.1. Weigh appropriate amount of SKG into 500 ml screw-cap flask.
 - 3.2. Add appropriate amount of 0.5X TBE prepared according to tables above; swirl gently to disperse agarose.
 - 3.2.1. Mix 1.0 g agarose with 100 ml 0.5X TBE for 14cm-wide gel form (10 wells)
 - 3.2.2. Mix 1.5 g agarose with 150 ml 0.5X TBE for 21cm-wide gel form (15 wells)
 - 3.3. Loosen cap or cover loosely with clear film and microwave for 60 seconds; mix gently and repeat for 15 second intervals until agarose is completely dissolved.
 - 3.4. Return flask to 55-60°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

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Loading Restricted Plug Slices on the Comb (Option 1)

1. 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. Alternatively, digested plug slices can be kept in the refrigerator for up to three days if they are stored in 0.5X TBE.

2. Assemble gel form, place 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. Make sure the comb teeth touch the gel platform, when comb is upright.
3. Remove plug slices from tubes; lay comb flat on gel form and load plug slices on the bottom edge of the teeth, including *Salmonella* serotype Braenderup H9812 standards in outside lanes (teeth) and between samples as needed (no more than 4 samples between standards).
4. Load samples on remaining teeth of the comb and note locations.
5. Remove excess buffer with a kimwipe. Allow plug slices to air dry on the comb for 3-5 minutes or seal them to the comb with 1% SKG agarose (55-60°C).
6. 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.
7. Carefully pour the agarose (cooled to 55-60°C) into the gel form and remove any bubbles or debris.
8. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE (2 L for CHEF-DR II). Close cover of unit.
9. 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.
10. Remove comb after gel solidifies, about 30-45 minutes.
11. 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.

Loading Restricted Plug Slices into the Wells (Option 2)

1. 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. Alternatively, digested plug slices can be kept in the refrigerator for up to three days if they are stored in 0.5X TBE.
2. 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.
3. 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.
4. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit.
5. Turn on power supply, pump calibrated to a flow rate of 1 liter/minute (setting about 70), and cooling module (14°C) approximately 30 minutes before gel is to be run.
6. Remove comb after gel solidifies for at least 30 minutes.
7. Remove plug slices from tubes with tapered end of spatula and load into appropriate wells, including *Salmonella* serotype Braenderup H9812 standards in outside lanes and between samples as needed (not more than 4 samples between standards). Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that there are no bubbles.

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.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55-60°C). Allow to harden for 3-5 minutes. 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

Listeria monocytogenes strains restricted with *Ascl* or *Apal*:

- 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 17-20 hours (See note below)
- 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: 17-20 hours (See note below)
- 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: 17-20 hours (See note below)

The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. The 21 cm wide (15-well) gels require ~1 hr longer than 14 cm wide (10-well) gels. 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.

Make note of the initial milliamp (mA) reading on the instrument. The initial mA should be between 110-150 mA. 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 an Agarose Gel

The following staining procedure describes the use of ethidium bromide to stain PFGE gels. Alternate DNA stains may be used. Please see the QuickTip ["20140218 Staining"](#) within the Library of PulseNet Documents forum (QuickTips/Wet Lab/PFGE) on the SharePoint site for additional information.

1. When electrophoresis run is over, turn off equipment; remove and place gel in an approximately 14 cm x 24 cm covered container with 400 ml ethidium bromide or other approved stain (see PNL02). Larger or smaller volumes may be used for different sized containers. Stain gel, gently rocking for 20-30 min in covered container.

*Ethidium bromide (EtBr) is toxic and a mutagen. Stock solutions of 10 mg/ml EtBr in water are available from several commercial companies (see PNL01). The diluted solution can be kept in the dark at room temperature and re-used up to 15 times within 3 weeks before discarding according to your institution's guidelines for hazardous waste; **do not pour down the drain**. Aqueous solutions containing EtBr can be treated using de-staining bags from Amresco (E732-25), which effectively and safely remove EtBr from solutions. Once the EtBr is removed, the treated aqueous solutions can be discarded down the drain. Refer to the Safety Data Sheets (SDS) provided by the vendor for more details.*

Currently, the acceptable alternative stain options are GelRed™ (Biotium, 41002), SYBR® Safe (ThermoFisher Scientific S33102) and SYBR® Gold (ThermoFisher Scientific S11494). 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 de-staining steps should be omitted. However, the gels can be briefly rinsed with CLRW before imaging. Diluted GelRed™ solution can be kept in the dark at room temperature and re-used up to 10 times within 10 days before discarding down the drain. Usage parameters have not been established for other alternative stains.

2. De-stain gel in approximately 500 ml CLRW for 60-90 min, changing water every 20 minutes (at least 3 times). Capture image on GelDoc XR, XR+ or equivalent documentation system. If background interferes with resolution, de-stain for an additional 30-60 min (2 or 3 more washes).
3. Follow directions given with the imaging equipment to save gel image as a *.1sc (QuantityOne software) or *.scn (ImageLab software) 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 software). Additional instructions are provided in PNL07 (Image Acquisition) 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.

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.

CLIA Laboratory Procedure Manual Requirements

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.

Records Management

1. Establish a record book or electronic log (for an example, see Appendix PNL04-1: PFGE Isolates and Plug Run Log) for tracking isolates, including but not limited to, the following information:
 - 1.1. Sample number (state public health lab identifier or similar)
 - 1.2. Date the isolates were received in the PFGE lab
 - 1.3. Date the plugs were made
 - 1.4. Location (box and slot) where the plugs are kept
 - 1.5. Gel and lane numbers for each sample
2. Establish a record (for an example, see Appendix PNL04-2: PFGE Plug Preparation Worksheet) of plug preparation, including but not limited to, the following information:
 - 3.1 Organism and number of samples
 - 3.2 Date plugs were made and initials of who made them
 - 3.3 Lot number and expiration dates of reagents used
3. Establish a record (for an example, see Appendix PNL04-3: PFGE Enzyme Master Mix and Gel Setup Worksheet) for each gel, including but not limited to, the following information:
 - 3.1 Gel number and date gel run
 - 3.2 Electrophoresis equipment used
 - 3.3 Running conditions/times
 - 3.4 Lot number and expiration dates of reagents used
 - 3.5 Restriction temperatures and times
 - 3.6 Order of isolates on gel

Formulas of PFGE Reagents

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)

50 ml of 1 M Tris, pH 8.0

100 ml of 0.5 M EDTA, pH 8.0

100 ml 10% N-Lauroylsarcosine, Sodium salt (Sarcosyl) OR 10 g of N-Lauroylsarcosine, Sodium salt (Sarcosyl).

Dilute to 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.

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 about 2 hours to completely dissolve the Sarcosyl.

Contacts

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Amendments

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 *Apal* (50 U/sample) and *Ascl* (40 U/ sample) for two to three hours
2. 2011-07: A note was added to clarify which buffers to use during the restriction digestion “step 2” under the enzyme mixture charts.
3. 2013-03 changes:
 - Corrected formula for TE buffer. TE used at CDC is 10mM 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 *Ascl* and *Apal* was reduced from 40 units 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.
- 8.4 2017-03 changes:
 - Reformatted to fit QA/QC SOP numbering scheme.
 - Updated vendor and contact information and added website link for SharePoint
 - Added Definitions and Terms
 - Revised Enzyme Calculations Table and TBE Calculations table.
 - Reformatted header and removed footer in accordance with new document layout.
 - Added Records Management Section; merging PNL08.
 - Added Appendices PNL04-1 through PNL04-3
 - Added “Approval Signature” section.

Appendix PNL04-3: PFGE Enzyme Master Mix and Gel Setup Worksheet Example

Purpose: _____
 Gel #: _____ Isolates Received from: _____
 Date Gel Run: _____

Place gel image here

	Set Temp	Actual Temp	Start time	End time
XbaI Digestion	37°C			
AscI Digestion	37°C			
ApaI Digestion	25°C			

Volt. Grad.	6V/cm
Incl. Angle	120°
Ramping	Linear
Low MW	N/A
High MW	N/A
Run Time	
Initial mA	
Mapper	
Delayed start?	<input type="checkbox"/>

Roche	Buffer H	
	1X	2
Water	180	360
10X Buffer	20	40
Total	200	400

NEB	CutSmart Buffer	
	1X	2
Water	180	360
10X Buffer	20	40
Total	200	400

Roche	XbaI - 50U	
	1X	2
Water	174	348
10X Buffer H	20	40
BSA (20mg/ml)	1	2
Enzyme (10U/μl)	5	10
Total	200	400

NEB	AscI - 25U	
	1X	2
Water	176.5	353
10X CutSmart Buffer	20	40
BSA (20mg/ml)	1	2
Enzyme (10U/μl)	2.5	5
Total	200	400

NEB	ApaI - 25U	
	1X	2
Water	178.5	357
10X CutSmart Buffer	20	40
BSA (20mg/ml)	1	2
Enzyme (50U/μl)	0.5	1
Total	200	400

	Lot Number	Exp date
SeaKern Gold		
BSA		
XbaI		
AscI		
ApaI		
10X Buffer H		
10X CutSmart Buffer		
10X TBE Buffer		
GelRed/Ethidium Bromide		

Well	Isolate	Buffer	Enzyme	Comments / State ID / Serotype	Plugs made (initial, date)
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					