

**STANDARD OPERATING PROCEDURE FOR PULSENET PFGE OF *CLOSTRIDIUM*
*BOTULINUM***

CODE: PNL25

Effective Date:

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1. **PURPOSE:** To describe the standardized protocol for molecular subtyping of *Clostridium botulinum* by Pulsed-field Gel Electrophoresis (PFGE).
2. **SCOPE:** To provide the PulseNet participants with the same procedure for performing PFGE of *Clostridium botulinum* thus ensuring interlaboratory 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:

BIOSAFETY WARNING:

All samples received must be considered infectious. *Clostridium botulinum* or its associated toxins may be present in a variety of food products, clinical materials (serum, feces) and environmental samples (soil, surface water). Exposure to the toxin of *C. botulinum* is the primary laboratory hazard. The toxin may be absorbed after ingestion or following contact with the skin, eyes, or mucous membranes, including the respiratory tract. Accidental parenteral inoculation may also represent a significant exposure to toxin. Broth cultures grown under optimal conditions for toxin production may contain 2,000,000 mouse LD(50) per ml of toxin.

Recommended Precautions: At a minimum Biosafety Level 2 practices, containment equipment and facilities are recommended for all activities with materials known to or that may potentially contain botulinum toxin. Personal protective equipment (PPE) i.e., gloves, lab coat, safety glasses and/or face shield should be worn at all times when handling anything that has come into contact with the organism or toxin including pipette tips and plastic transfer pipettes used to transfer liquids. All liquids require disinfection using a freshly prepared 10% bleach solution. Solutions of sodium hypochlorite (0.1%) or sodium hydroxide (0.1N) readily inactivate the toxin and are recommended for decontaminating work surfaces and spills of cultures or toxin. Additional primary containment and personnel precautions, such as those recommended for Biosafety Level 3, are indicated for activities with a high potential for aerosol or droplet production, and those involving production quantities of toxin. Animal Biosafety Level 2 practices, containment equipment and facilities are recommended for diagnostic studies and titration of toxin. A pentavalent (ABCDE) botulism toxoid is available through the Centers for Disease Control and Prevention, as an investigational new drug (IND). This toxoid is recommended for personnel working with cultures of *C. botulinum* or its toxins.

Please read all instructions carefully before starting protocol. All plasticware, glassware, pipets, spatulas, etc. that come in contact with the cell suspensions or plugs should be disinfected with 10% bleach for at least 1 hour before they are washed and reused.

SELECT AGENTS REQUIREMENTS:

All PFGE materials, including restricted plugs, may contain viable bacteria until the start of electrophoresis. Since *C. botulinum* is a Select Agent (SA), and according to the APHIS/CDC SA Regulations (7 CFR Part 331 and 9 CFR Part 121), all parts of the following procedure, except electrophoresis, must be performed by SA approved personnel and within SA approved space. In addition, long term stored plugs may be subject to SA inventory requirements, as defined by APHIS/CDC SA Regulations 42 CFR Part 73.17 (1), 7 CFR Part 331.17(1) and 9 CFR Part 121.17(1).

Please refer to <http://www.selectagents.gov/Regulations.html> for additional information.

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PREPARATION OF PFGE PLUGS FROM TEST CULTURES

Day 1

1. Streak each test culture for colony isolation onto Anaerobic Blood Agar Plate – CDC formulation (ANA-BAP).
2. Incubate the plates in an anaerobic chamber overnight at 37°C ± 2°C.

Day 2

1. Label small tubes (12-mm x 75-mm Falcon tubes or equivalent) with culture numbers.
2. Prepare **Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0)** as follows:

200 ml of 1 M Tris-HCl, pH 8.0

400 ml of 0.5 M EDTA, pH 8.0

Dilute to 2000 ml with sterile Ultrapure water (Clinical Laboratory Reagent Water (CLRW))

Note: Acceptable options for CLRW is type I or Milli-Q water.

3. Transfer ≈1.5 ml of **Cell Suspension Buffer (CSB)** to the labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from the ANA-BAP plate; suspend cells in CSB by spinning the swab gently so cells will be evenly dispersed and formation of aerosols is minimized.
4. Adjust concentration of cell suspensions to a milky turbidity of ~1 McFarland (0.18-0.20 on Dade Microscan Turbidity Meter) by diluting with sterile CSB or by adding additional cells.
5. Pipette 1000 µl of the cell suspensions into sterile microcentrifuge tubes and spin for 5 minutes at 5,000 rpm.
6. Remove the supernatants, resuspend cells in 1000 µl of the cell suspension buffer and spin for 5 minutes at 5,000 rpm.
7. Remove the supernatants and re-suspend cells in 500 µl of the cell suspension buffer.
8. Use these washed cells to inoculate two EYA plates per sample for confluent growth (250 µl per plate), using the Kirby –Bauer technique.
9. Incubate the plates for 18-72 hours in an anaerobic chamber at 37°C ± 2°C.

Note: Remove plates from incubation as soon as there is sufficient growth for testing. 18 hours is typically optimal.

Day 3

1. Turn on shaker water bath or incubator (54°C ± 2°C) and stationary water baths (55°C ± 2°C and 37°C ± 2°C).
2. Add bottles of sterile Ultrapure water (CLRW) and TE buffer to the 55°C water bath to warm for the washing procedural steps.

Note: Acceptable options for CLRW is type I or Milli-Q water.

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3. Prepare **2X Cell Lysis Buffer (12mM Tris, 2M NaCl, 200 mM EDTA, 1% Brij 58, 0.4% Deoxycholate, 1% Sarkosyl)** as follows:

1.2 ml of 1 M Tris-HCl, pH 8.0
40 ml of 5 M NaCl
40 ml of 0.5 M EDTA, pH 8.0
1 g Brij 58
0.4 g Deoxycholate
5 g Sarkosyl or 50 ml of a 10% Sarkosyl Solution
Dilute to 100 ml with sterile Ultrapure water (CLRW)

Note: Acceptable options for CLRW is type I or Milli-Q water.

4. Prepare Lysozyme (Sigma L7651 or equivalent) stock solution (20 mg/mL in TE) as follows:
- Weigh out 100 mg Lysozyme (keep the container of Lysozyme on ice)
 - Add 5 mL TE buffer, swirl to mix
 - Aliquot 250 uL amounts into small eppendorf tubes and freeze for future use.

5. Prepare Mutanolysin (Sigma M9901 or equivalent) stock solution (5 U/ μ L in TE) as follows:
- Add 1 mL TE buffer to vial of lyophilized Mutanolysin, swirl to mix
 - Aliquot 50 μ L amounts into small eppendorf tubes and freeze for future use.

6. Take out tubes of Lysozyme (20 mg/ml) and Mutanolysin (5U/ μ l) needed from the -20°C freezer and pre-warm **2X Cell Lysis Buffer** to 55 °C \pm 2°C.

7. Prepare 1.2% SeaKem Gold agarose in **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)** for PFGE plugs as follows:

- Weigh 0.12 g (or 0.24 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask
- Add 10.0 ml (or 20.0 ml) TE Buffer; swirl gently to disperse agarose.
- 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.
- Recap flask and place in 55°C \pm 2°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.

8. Label small tubes (12-mm x 75-mm Falcon tubes or equivalent) with culture numbers.
9. Prepare **PIV Buffer** as follows:

5 ml of 1 M Tris-HCl, pH 8.0
100 ml of 5 M NaCl
Dilute to 500 ml with sterile Ultrapure water (CLRW)

Note: Acceptable options for CLRW is type I or Milli-Q water.

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- Transfer ≈1.5 ml of **PIV Buffer** to the labeled tubes. Use a sterile polyester-fiber or cotton swab that has been moistened with sterile PIV to remove some of the growth from the agar plate; suspend cells in PIV by spinning swab gently so cells will be evenly dispersed and formation of aerosols is minimized.

Note: If a large number of samples are being prepared, it is recommended that they are prepared in batches of ~10 samples at a time.

- Adjust concentrations of cell suspensions to 0.68-0.72 as measured on the Dade Microscan Turbidity Meter by diluting with sterile PIV Buffer or by adding additional cells. These cell suspension concentrations give satisfactory results at CDC; each laboratory may need to establish the optimal concentration needed for satisfactory results.

Note: Cell suspensions need to be at room temperature when concentration is checked.

- Pipette 1000 µl of the cell suspensions into microcentrifuge tubes and spin for 5 minutes at 5,000rpm.

- Calculate the volume of 2X cell lysis buffer and Proteinase K required per sample as follows:

Note: Stock concentrations of Proteinase K may vary by lot and will need to be calculated for each shipment. Adjust total volume to compensate by changing amount of 2X cell lysis buffer used. The final concentration of Proteinase K for plugs is 0.665 mg/ml.

Example Calculation:

(stock conc. of Proteinase K) (X) = (final conc. desired) (final volume)

(stock conc. of Proteinase K) (X) = (0.665 mg/ml) (1 ml)

$\frac{(0.665 \text{ mg/ml}) \times (1 \text{ ml})}{(\text{stock conc. of Proteinase K})} = \text{volume of Proteinase K needed (ml)}$

Total volume of 2X cell lysis buffer + Proteinase K = 316 µl

316 µl – volume of Proteinase K = volume of 2X cell lysis buffer needed (µl)

- Remove the supernatants and resuspend cells in the calculated volume of 2X cell lysis buffer that has been pre-warmed to 55°C ± 2°C. Then add 80 µl of lysozyme (20 mg/ml) and incubate in the waterbath for 20 minutes at 55°C ± 2°C.
- After removing the samples from the waterbath, add 4 µl of mutanolysin (5U/µl) and the calculated volume of proteinase K, and incubate another 10 minutes at 37°C ± 2°C.

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!

- Add 400 µl (0.4 ml) melted 1.2% SeaKem Gold agarose to the cell suspensions; mix by gently pipetting mixture up and down a few times. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60°C).

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- 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.

Note: If plugs are being prepared from a large number of samples, it is recommended that they are prepared in batches of ~10 samples at a time.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs of the same strain can be lysed in the same 50-ml tube.

- Label 50-ml polypropylene screw-cap tubes with culture numbers.
- Prepare **ES Buffer** as follows:
495 ml of 0.5 M EDTA, pH 8.0
5 ml of 10 % Sarcosyl
- Add 5 ml of ES Buffer to each labeled 50 ml tube.
- Calculate the volume of **Proteinase K** needed per sample as follows:

Note: Stock concentrations of Proteinase K may vary by lot and will need to be calculated for each shipment. The final concentration of Proteinase K per tube is 0.14 mg/ml.

Calculation:

$$(\text{stock conc. of Proteinase K}) (X) = (\text{final conc. desired}) (\text{final volume})$$

$$(\text{stock conc. of Proteinase K}) (X) = (0.14 \text{ mg/ml}) (5 \text{ ml})$$

$$\frac{(0.14 \text{ mg/ml}) \times (5 \text{ ml})}{(\text{stock conc. of Proteinase K})} = \text{volume of Proteinase K needed (ml)}$$

Add the calculated volume of Proteinase K to each labeled 50 ml tube containing ES Buffer.

- 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 tubes containing ES Buffer + Proteinase K. Be sure plugs are under buffer and not on side of tube.

Note: Ensure that the green screen caps are in place on conical tubes containing the plugs to prevent the loss or damage of the plug

- Place both sections of the plug mold, spatulas, and scalpel in 10% bleach. **Soak them for 1 hour before washing them.**
- Place tubes in a rack and incubate in the 54°C ±2°C shaker water bath or incubator for a minimum of 2 h with constant agitation (40-70 rpm). Be sure water level is above level of lysis buffer in tubes if using a water bath.

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.

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1. Remove tubes from water bath and carefully pour off ES Buffer into an appropriate discard container; plugs can be held in tubes with the green screen caps.

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 15 ml sterile Ultrapure water (CLRW) that has been preheated to $55^{\circ}\text{C} \pm 2^{\circ}\text{C}$ to each tube and shake the tubes in a $54^{\circ}\text{C} \pm 2^{\circ}\text{C}$ water bath for 15 min at 70 RPM.

Note: Acceptable options for CLRW is type I or Milli-Q water.

3. Pour off water from the plugs and repeat wash step with 20 ml of preheated water (Step 2) one more time.
4. Pour off water, add a minimum of 20 ml preheated ($55^{\circ}\text{C} \pm 2^{\circ}\text{C}$) sterile TE Buffer, and shake the tubes in the $54^{\circ}\text{C} \pm 2^{\circ}\text{C}$ water bath for 15 min at 70 RPM.
5. Pour off TE and repeat wash step with preheated TE five more times.
6. Decant last wash and add 15 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 containing 1 ml of TE for storage.

Day 4

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS

Note: A small slice of the plug or the entire plug (made in disposable plug molds) can be digested with the restriction enzyme. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes. Restriction analysis with a secondary enzyme is important in situations where the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable.

1. Label 1.5 ml microcentrifuge tubes with sample numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812 standards.
2. Prepare 1X restriction buffer by diluting the appropriate 10X restriction buffer (provided with each restriction enzyme by the vendor 1:10 with sterile Ultrapure water (CLRW) according to the following tables:

Note: Acceptable options for CLRW is type I or Milli-Q water.

***Sma*I digestion buffer**

Reagent	$\mu\text{l}/\text{Plug Slice}$
Sterile Ultrapure Water	180 μl
Restriction Buffer 4	20 μl
Total Volume	200 μl

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***Xba*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	180 µl
Restriction Buffer H	20 µl
Total Volume	200 µl

***Xho*I digestion buffer**

Reagent	µl/Plug Slice
Sterile Ultrapure Water	160 µl
Restriction Buffer 4	20 µl
10X BSA	20 µl
Total Volume	200 µl

Note: Use buffer H (Roche) for the standard plugs and buffer 4 (New England Biolabs) for the sample plugs. Buffer 4 requires the addition of BSA when the plugs are going to be digested with *xho*I.

3. Add 200 µl diluted restriction buffer (1X) to the corresponding (samples or standards) 1.5 ml microcentrifuge tubes.
4. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.
5. Cut a 2.0 to 2.5 mm wide slice from test sample plugs with a single edge razor blade (or scalpel, coverslip, etc.) and transfer to tube containing appropriate diluted restriction buffer. Be sure plug slice is under buffer. Replace rest of plug into the original tube that contains TE buffer. Store at 4°C.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. 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). 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).

6. Cut three or four 2.0 to 2.5 mm wide slices from plug of the *S. ser. Braenderup* H9812 standard and transfer to tubes with diluted restriction buffer H. Be sure plug slices are under buffer. Replace rest of plug in original tube that contains TE buffer. Store at 4°C.

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7. Incubate sample and standard plug slices in 37°C water bath (for plugs that will be digested with *XbaI* or *XhoI*) or 25°C water bath (for plugs that will be digested with *SmaI*) for 10 minutes.
8. Prepare a restriction enzyme master mix with the appropriate restriction enzyme per sample according to the following tables. Prepare enough for each plug slice plus one additional aliquot.

***SmaI* (10U/μl stock concentration)**

Reagent	μl/Plug Slice
Sterile Ultrapure Water	175 μl
10X Restriction Buffer 4	20 μl
Enzyme (50 U/sample)	5 μl
Total Volume	200 μl
Incubate	25°C, 4 hours

***XbaI* (20U/μl stock concentration)**

Reagent	μl/Plug Slice
Sterile Ultrapure Water	177 μl
10X Restriction Buffer H	20 μl
Enzyme (60 U/sample)	3 μl
Total Volume	200 μl
Incubate	37°C, 2 hours

***XhoI* (20U/μl stock concentration)**

Reagent	μl/Plug Slice
Sterile Ultrapure Water	155 μl
10X Restriction Buffer 4	20 μl
10X BSA	20 μl
Enzyme (100 U/sample)	5 μl
Total Volume	200 μl
Incubate	37°C, 3 hours

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Note: Digest *Salmonella* standard plugs with Roche *XbaI* and sample plugs with New England Biolabs *SmaI* or *XhoI*. These restriction enzymes give satisfactory results at CDC. Restriction enzymes provided by other vendors have not been evaluated. Be certain that the restriction buffer that is used is recommended by the vendor for the corresponding restriction enzyme and calculate the volume of enzyme needed to achieve the same final concentrations as indicated in the tables. Keep vials of restriction enzymes on ice or in insulated storage box (-20°C) at all times.

- a. **Addition of Bovine Serum Albumin (BSA):** Several restriction enzyme vendors specifically recommend the addition of 1X BSA to enzyme restriction mixtures. However, BSA can be added to all enzyme restriction mixtures and may assist in reducing the incidence of incomplete restriction. If BSA is added to the enzyme reaction mixture, the volume of BSA added should be deducted from the volume of water to maintain the total volume of 200 µl per slice.
9. 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.
10. Add 200µl of the appropriate restriction enzyme cocktail to each of the tubes, making sure the plug is completely submerged in the solution. Close the tubes and mix by gently tapping.
11. Incubate the plug slices with restriction enzymes at each of their respective temperatures and times.

LOADING PFGE PLUG SLICES AND POURING AGAROSE GEL

1. **Confirm that water bath is equilibrated to 55°C ±2°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)					
	200	210	220	230	240	250
5X TBE	200	210	220	230	240	250
Reagent Grade Water¹	1800	1890	1980	2070	2160	2250
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

10X TBE:

Reagent	Volume in milliliters (ml)					
	100	105	110	115	120	125
10X TBE	100	105	110	115	120	125
Reagent Grade Water	1900	1995	2090	2185	2280	2375
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

¹ De-ionized water (does not need to be sterilized).

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2. 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.
 - c. Loosen or remove cap and cover loosely with clear film, 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°C ±2°C water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 wells)

Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (15 wells)

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

4. Place the gel mold 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 baths. 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.
7. Remove excess buffer with tissue. Allow plug slices to air dry on the comb for a minimum of 15 minutes.
8. Position comb in gel mold and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform, and there are no bubbles.
9. Carefully pour the agarose (cooled to 55°C ± 2°C) into the gel mold and allow the gel to solidify for a minimum of 15 minutes at room temperature.
10. Put black gel frame in electrophoresis chamber and add 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 cooling module (14°C), power supply, and pump (set at ≈70 to achieve a flow rate of 1 liter/minute).
12. After the gel has solidified, add 5-10 ml of 0.5X TBE to the comb and gently remove from the gel.
13. Unscrew and remove end gates from gel mold; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber.
14. Add 860 µl of Thiourea (10mg/ml) to 0.5X TBE in the electrophoresis chamber. Close cover of chamber.

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ELECTROPHORESIS CONDITIONS

1. Select the following conditions on the CHEF MAPPER:
 - a. Select the following conditions on the CHEF MAPPER
 Auto Algorithm
 30 kb - low MW
 600 kb - high MW
 Initial switch time: 0.5 s
 Final switch time: 40 s
Change run time to 18 - 19 h (See note below)
 (Default values: Initial switch time = 2.16 s; Final switch time = 54.17 s)
 - b. Select following conditions on **CHEF-DR III**
 Initial switch time: 2.2 s
 Final switch time: 54.2 s
 Voltage: 6 V
 Included Angle: 120°
 Run time: 18-19 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. Make note of the initial milliamp (mAmp) reading on the instrument. The initial mAmps should be between 110-170 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 5

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide. Dilute 50 µl of ethidium bromide stock solution (10 mg/ml) with 500 ml of reagent grade water (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 minutes in covered container with gentle agitation.

Note: Use extreme caution when handling Ethidium Bromide, as it is carcinogenic and a mutagenic chemical. 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). 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.

2. Destain gel in approximately 500 ml reagent grade water for 60 min; changing water every 20 minutes. Capture image using a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system. If too much background is observed destain for an additional 30-60 min.

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a. Alternative Method for PFGE Gel Staining (GelStar): Dilute 40 ul of GelStar (10,000X stock solution) into 400 ml of 1X TBE. Stain gel for 60 minutes in covered container with gentle agitation. Capture image using a Gel Doc 1000, 2000, EQ, or XR, or equivalent documentation system.

Note: Destaining is not necessary when staining the gel with GelStar. After staining, proceed to capturing the image with a gel documentation system. Stock solutions of GelStar are available from Lonza, 50535. **Use the same precautions when handling and disposing of GelStar as indicated above for EtBr.**

3. Follow directions given with the imaging equipment to save gel image as an *.img or *.lsc 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. Additional instructions are provided in PNL07 of the PulseNet QA/QC manual.
4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2-4 L reagent grade water 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. Remove and clean loose pieces of agarose in the electrophoresis chamber.
5. If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

Please note the following if PFGE results do not have to be available within 24-28 hours:

1. Plugs can be lysed for longer periods of time (3-16 hours).
2. 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.
3. The restriction digestion can be done for longer periods of time (3-16 hours).

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

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.

5. FLOW CHART:

6. BIBLIOGRAPHY:

7. CONTACTS:

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8. AMENDMENTS:

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