Unified Pulsed-Field Gel Electrophoresis (PFGE) Protocol for Gram Positive Bacteria

Introduction: This PFGE protocol had been developed to unify and streamline the molecular strain typing of several clinically-important, Gram-positive pathogens under routine surveillance or investigation by the Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention. It is a rapid, one-day protocol that has been validated for a range of *Staphylococcus* spp., *Enterococcus* spp., and *Clostridium* spp., including *Clostridium difficile*, among others. The development of a unified PFGE protocol for these organisms is intended to simplify laboratory training, proficiency testing, and the tracking of reagents and consumables, and to limit both the cost and the likelihood of systematic procedural errors due to the maintenance of multiple, highly-similar protocols. For external proficiency testing and validation, challenge sets are offered for *Staphylococcus aureus* (BSTS) and *Enterococcus* spp. (BSTE) through the College of American Pathologists.

Materials and Equipment

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

Equipment	Reagents & Media	Supplies
1. Siemens MicroScan™ Turbidity Meter (Siemens Healthcare Diagnositics, Tarrytown NY) 2. 37°C Incubator 3. Shaking incubator 4. 55°C Water bath 5. Screened Caps (Bio-Rad™ 170-3711) 6. Analytical balance 7. Reusable Plug Mold (Bio-Rad™ 170-3622) 8. 21x14cm Casting Stand (Bio-Rad™ 170-3704) 9. Leveling Table (Bio-Rad™ 170-4046) 10. Combination Comb Holder (Bio-Rad™ 170-3699) 11. 15-well Comb (Bio-Rad™ 170-3627) 12. Bubble Level 13. 20, 200 and 1000µL Micropipettes 14. Bio-Rad™ CHEF Mapper, CHEF Mapper XA, DR-II or DR-III electrophoresis system, including cooling module and pump.	1. Anaerobic Blood Agar (Clostridium spp.) or Trypticase Soy Agar with 5% Sheep's Blood 2. Recombinant Lysostaphin (1mg/mL in 20mM sodium acetate) 3. Lysozyme (20mg/mL in TE) 4. Proteinase K (20mg/mL) 5. SeaKem® Gold (Lonza, Rockland ME)) or Agarose III (Amresco LLC, Solon OH) 6. Reverse osmosis water 7. ATSM Type I or HPLC grade water 8. Tris-EDTA (TE) buffer (10mM, pH 8.0) 9. 10X Tris-Borate EDTA buffer 10. Thiourea (0.52M; 1g:25mL) 11. Ethidium bromide 12. Cell Lysis Buffer (50mM Tris: 50mM EDTA: 1% sodium sarcosyl)	1. 1.5mL microfuge tubes, sterile 2. BD™ Falcon 2054 Tubes 3. BD™ Falcon 50mL Conical Tubes 4. Microtube rack 5. Sterile, filtered pipette tips for P20, P200 and P1000 6. Kimwipe™ lint-free tissue 7. 125mL Erlenmeyer flask with cap 8. 500mL Erlenmeyer flask with cap 9. 25cm x 25cm gel staining tray

Procedure

A. Preparation of Agarose Plugs

1. Inoculate pure isolate onto an appropriate, non-selective agar medium, such as trypticase soy agar with 5% sheep's blood (eg, TSA II, BD Microbiology Systems, Sparks, MD), or anaerobic blood agar (ana-BAP), and incubate the plate overnight to ensure adequate growth. All isolates for PFGE should be processed from 24 hour, pure cultures on an appropriate non-selective medium. When processing *Clostridia* and other anaerobes, care should be taken to limit the exposure of these culture plates to environmental oxygen.
2. Dispense 2mL Tris-EDTA (TE) buffer (pH8, 0.01M) into sterile BD Falcon 2054 (12 x 75mm) snap-top tubes. Prepare one extra tube to be used as a blank for optical density (OD) readings.
3. Using a sterile, cotton-tipped applicator swab, gently sweep bacteria from each overnight purity plate, and prepare standardized suspensions using a Siemens Microscan turbidity meter. For Staphylococci and Enterococci, use a starting OD of 0.6. For *Clostridium*, use a higher OD of 0.8 to 1.0. Label each tube carefully to avoid handling errors.
4. Once the standardized suspensions are made, prepare a sufficient number of sterile, 1.5mL microfuge tubes as follows: Carefully place the sterile microtubes into a rack, and dispense 20µL (20mg/mL) of lysozyme into each tube. Lysozyme should be freshly prepared from stock, and excess should be discarded after use. For Staphylococci, also add 5µL of recombinant lysostaphin to each tube.
5. Promptly transfer 400µL of the first bacterial suspension into the first 1.5mL microfuge tube, close and label it accordingly. Repeat this process for the remaining bacterial samples.
6. Place the microtubes into a foam or plastic floating rack and incubate for 30 to 45 minutes in a 55ºC water bath. Deposition of cellular debris on the sides and bottom of the microtube are normal following this pre-lysis step.
7. Prepare a 1.0 to 1.2% (w/v) agarose gel in TE buffer. Weigh 0.25 to 0.30g of SeaKem® Gold (Lonza, Walkersville, MD), and add it to a clean, dry 125mL Erlenmeyer flask. Add 25mL of TE Buffer (0.01M; pH8.0), and swirl gently to remove clumps. Microwave on high for 30 seconds, swirling occasionally. Do not over boil. Remove flask of molten agarose from the microwave and place it in a 55°C waterbath, using a weighted ring for stability.

   Note 1: Amresco Agarose III (Amresco Inc, Solon, OH) may be substituted for SeaKem® Gold, but a 1.2% concentration is advisable due to differences in tensile strength.

   Note 2: Agarose concentration used for the plugs is largely a matter of preference: higher concentrations are easier to manipulate, but may require additional TE washes to ensure the complete removal of detergents and lysis reagents.

8. While the plugs are setting, prepare and label large tubes for the lysis step. The use of 50mL conical screw-top tubes and green BioRad™ screen caps is strongly recommended, particularly if large numbers of samples are to be run. Add 5mL of Simplified Gram Positive Lysis Buffer and 25µL (20mg/mL) Proteinase K to each tube and label them carefully.
9. Dispense plugs into corresponding lysis tubes. Incubate at 54ºC for at least two hours with vigorous (150-175RPM) agitation.
10. Wash the plugs twice with 10mL of reverse osmosis (or sterile) water for 10 to 15 minutes each. Then wash the plugs three times with 10mL of TE buffer for 10 to 15 minutes each. All washes should be

performed at room temperature, with constant 150-175RPM agitation. Inspect the plugs carefully, and include an additional wash step if detergents appear to persist.

11. Plugs may be stored in fresh TE buffer for up to six months at 4°C.

B. Restriction of Agarose Plugs

1. Using a metal spatula, remove each plug from TE buffer, and slice a 1 to 1.5mm slice using a straight razor blade. If available, a notched plexi-glass cutting stand is extremely helpful to ensure consistency. If a cutting stand is not available, the use of an empty petri dish or other sterile, non-porous surface is recommended to preserve plug integrity. Cutting and restricting duplicate slices for each specimen is a recommended practice, as it will facilitate repeat testing in the event of a failed gel.
2. Dispense the two 1 to 1.5mm plug slices into a sterile 1.5mL microfuge tube. Repeat this process for the remaining samples. For a 15 well gel, 12 samples may be run.
3. Prepare a thirteenth microtube for the *Salmonella* Braenderup H9812 universal standard. Cut three slices of an H9812 stock plug, and transfer them to the microtube.
4. Prepare a restriction mastermix for all 12 sample tubes, as well as the H9812 standard. Each microtube will contain:

    175µL of molecular-grade water
    20µL of an appropriate 10X restriction buffer
    2µL Bovine serum albumin (BSA)
    3µL of an appropriate restriction endonuclease

    Thus, for 12 sample lanes, the mastermix should contain 2100µL water, 240µL buffer, 24µL BSA, and 36µL of an appropriate restriction enzyme.

    The choice of restriction endonuclease depends on the genomic composition of the organism that is to be tested. Default enzymes for most of the most common bacteria in our laboratory are included below. Consult vendor materials to determine appropriate 10X buffer and incubation parameters for each enzyme.

    Salmonella Braenderup H9812: *Xba*I

    Staphylococcus aureus: *Sma*I (*Eag*I secondary; *Cfr*9i isoschizomer)
    Clostridium difficile: *Sma*I (*Mlu*I or *Eag*I secondary)
    Enterococcus spp : *Sma*I (*Spe*I secondary)

5. Incubate samples and standard tubes for at least two hours to ensure complete restriction. Incubation parameters differ; please consult endonuclease product inserts and vendor resources for the most appropriate reaction conditions. Typically, *Cfr*9i, *Eag*I, *Mlu*I, and *Xba*I restrictions are all incubated at 37°C, while *Sma*I is incubated at 25°C.
6. Using a 1000µL micropipette, aspirate and discard the restriction mixture from each tube. Immerse the restricted plug slices in fresh TE buffer until they are ready to be loaded onto the gel. Restricted plug slices may be kept overnight in TE at 4°C.

C. Gel Preparation and Casting

1. Assemble the components required to cast a PFGE gel: casting stand and backplane, leveling table, bubble level, and comb. Assemble the casting stand and backplane, and place it on the leveling table. Place the bubble level in the center of the backplane, and adjust the leveling table until the bubble is centered. Position the comb in the empty tray, and ensure that it sits with the teeth completely level, in contact with the backplane. To prepare for sample loading, lay the comb down across the casting stand, so that the teeth are horizontal, and facing the user.
2. Prepare 150mL of a 1% (w/v) agarose gel, by weighing 1.50g of SeaKem® Gold (Lonza™) or Agarose III (Amresco™), and adding it to a clean, dry 500mL Erlenmeyer flask. Add 150mL of 0.5X Tris-Borate EDTA (TBE) buffer, and swirl gently to dislodge clumps.
3. Place the cap loosely on the Erlenmeyer flask, and microwave it on high for 1 to 1.5 minutes, swirling occasionally until agarose is completely dissolved. CAUTION: Do not over-boil; agarose may superheat and be prone to bumping. Remove molten agarose from microwave, and transfer it to a 55°C water bath until ready for use.
4. Using a metal spatula, carefully apply the restricted sample plug slices to teeth 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14 of the comb, and gently position them against the edge. Apply the restricted *Salmonella* Braenderup H9812 plug slices to the comb on teeth 1, 8 and 15.
5. Use the corner of a folded KimWipe (Kimberly-Clark, Roswell GA) to gently blot excess TE from each plug slice.
6. Carefully rotate the comb until it is upright, and set it into position in the notches on either side of the casting stand. Using a metal spatula, gently adjust the plug slices so that they make contact with both the tooth of the comb, and the backplane of the gel casting stand. Ensure that there are no air bubbles, and that the plug slices make complete contact with both surfaces. Allow the plugs to air dry in place for 5 minutes.
7. Remove molten agarose from 55°C water bath, and allow it to cool until it can be comfortably held. Pour the agarose into the casting stand, pouring slowly and steadily from a point near the center of the backplane. Take care to avoid introducing air bubbles or dislodging the plug slices from the comb. Air bubbles should be removed or moved to the margins using a metal spatula.
8. Allow the agarose to set for at least 15 minutes.

D. Gel Loading and Electrophoresis

1. Using a bubble level, ensure that the electrophoresis chamber is level, that all hoses are properly attached, and that the system is in good working order.
2. Place the black gel frame in the electrophoresis chamber, and ensure that it is properly seated.
3. Prepare 2 liters of 0.5X TBE by adding 100mL of 10X TBE to 1900mL in a 2000mL graduated cylinder. Mix gently.
4. Pour all 2000mL of 0.5X TBE into the electrophoresis chamber. For *Clostridium* spp. (or non-typable organisms), add 760µL of 0.52M thiourea to the running buffer. Caution: Thiourea is toxic, and should be handled with appropriate care.
5. Turn on the pump, followed by the chiller unit. Confirm that the chiller has defaulted to a 14°C set point, and allow buffer to circulate and cool completely before starting the run.

Note : Do not operate the chiller while the pumps are off. Without buffer circulating in the lines, the internal coolant lines in the chiller can freeze, leading to hardware failure and costly repairs.

6. Configure the PFGE controller using the following two-state electrophoresis parameters:

For *Clostridium* spp. and *Staphylococcus* spp.
Voltage density: 6.0V/cm
Included angle: 120°
Initial Switch Time: 5.0s
Final Switch Time: 40.0s
Run time: 19 to 20h
Ramping: Linear

For *Enterococcus* spp.
Voltage density: 6.0V/cm
Included angle: 120°
Initial Switch Time: 3.5s
Final Switch Time: 23.5s
Run time: 19 to 20h
Ramping: Linear

Consult the DR-II, DR-III or CHEF Mapper users' manual for specific instructions on system configuration.

7. Carefully remove the comb from the gel in a fluid motion, and unscrew the side panels on the casting stand. Gently lift the backplane from one side and remove the gel from the casting stand, taking care not to dislodge it or compromise its adherence to the backplane. Using a KimWipe, remove excess agarose from the bottom surface of the backplane, and carry the gel over to the waiting PFGE system.
8. Carefully place the gel into the chamber, sliding the backplane securely into the frame in the center of the electrophoresis chamber. Angle the gel slightly when submerging it to avoid trapping air bubbles underneath the gel.
9. Allow the gel to equilibrate for several minutes and come down to the ambient temperature of the surrounding buffer.
10. Press "START" to begin the run.
11. Place the completed PFGE worksheet on top of the electrophoresis chamber for reference. Record the starting current. For a standard gel with 2L of 0.5X TBE buffer, the starting current should register between 110 and 150mA.

E. Post-Staining and Imaging

1. Verify that the electrophoresis run has completed successfully, and document any errors that have registered on the controller display.

2. Open the electrophoresis chamber, and carefully remove the gel. Gently slide the gel off of the backplane and into a 25cm x 25cm gel staining tray. Agarose protrusions on the four corners of the gel that served as anchors will be sheared off; this is normal.
3. Pour Ethidium bromide post-stain over the gel to submerge it completely. Place on a rocker platform on low speed for 10 to 15 minutes.

   Note: Ethidium bromide post stain consists of 250µL of Ethidium bromide in 1L of water, and can be re-used up to 10 times. It must be stored away from ambient light to prevent photobleaching.
4. Decant off the Ethidium bromide post stain, and store it for re-use. If you are disposing of Ethidium bromide waste, follow the laboratory safety guidelines for Ethidium bromide handling and disposal.
5. Resubmerge the gel in reverse osmosis water and return it to the rocker platform for 30 minutes to an hour to allow for complete de-staining.
6. Image the gel according to the most current instructions for your gel documentation system software.

   Ensure that the gel is properly aligned and framed in the camera's field of view before activating the UV transilluminator. When taking your picture, take care not to over or under expose the image, and avoid prolonged UV exposure of the gel.

   Save the image in both the editable, native format for the gel documentation system, as well as a tagged image file format (TIFF). For BioNumerics (Applied Maths, Austin TX), the TIFF should be saved as a black and white image, with a color depth of 8 bits per pixel (8bpp).