



Oxacillin-resistant *Staphylococcus aureus* on PulseNet (OPN):
Laboratory Protocol for Molecular Typing of *S. aureus*
by Pulsed-field Gel Electrophoresis (PFGE)

MATERIALS

Supplies specified in the following protocol are those used at CDC and are itemized for purposes of reference identification. Equivalent materials may be substituted where appropriate, except for agarose, buffers, and media, which should be used as specified in the protocol.

Growing Cultures:

Test cultures inoculated on trypticase soy agar plates containing sheep blood

Loops

35-37⁰C shaking incubator or shaking water bath

BHI, 5 ml in 15 x 100 mm tubes (Remel¹ #060270, BD #22183)

Vortex mixer

Plug Preparation:

55-60⁰C stationary water bath

37⁰C stationary water bath

SeaKem Gold agarose (BMA² #50152)

TE Buffer (see Buffer Solutions, page 13)

Clean beaker for “working” TE buffer

Scale for measuring agarose

250 ml Erlenmeyer flask, screw-cap

Microwave

¹Use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

²BioWhittaker Molecular Applications, formerly FMC, Inc.



Sterile, Type I (reagent grade) water, about 50 ml, prewarmed to 55 C

Heat-resistant gloves

Lead ring weight for anchoring flask in water bath

1.5 ml sterile microcentrifuge tubes

17 x 100 mm polystyrene tubes (Falcon #2057) or equivalent

BHI, 5 ml in 15 x 100 mm tubes

Vortex mixer

Instrument to measure standardized cell suspensions:

MicroScan Turbidity Meter or Spectrophotometer and appropriate cuvettes

One 200 μ l and two 1000 μ l Pipetman or equivalent

Microcentrifuge

Glass capillary pipettes with bulb or attached to aspirator

Masking tape or equivalent for labeling plug mold

PFGE plug mold (Bio-Rad #170-3622 or #170-3713)

Lysostaphin stock solution (recombinant [Sigma #L-0761]

or conventional [Sigma # L-7386])

Plug Lysis:

Spatula to remove plugs from mold

5 ml pipettes

EC lysis buffer (see Buffer Solutions, page 13)

37⁰C stationary water bath

Plug Washing:

TE buffer (see Buffer Solutions, page 13)

Spatula or equivalent to hold plug in tube

5 ml pipettes

Orbital rocker or equivalent at room temperature

Restriction Enzyme Digestion:

Pretested *Salmonella* serotype Branderup strain H9812

1.5 ml microcentrifuge tubes

5 ml pipettes

One 200 μ l and one 1000 μ l Pipetman or equivalent

Commercial enzyme buffer appropriate for enzyme

Restriction enzyme (e.g., *Sma*I [Promega #R6125]; [New England Biolabs #R0141] *Xba*I

[Promega #R6185]; [New England Biolabs #R0145])

Sterile, Type I water



5-15 ml sterile plastic tube for preparing buffer-water and enzyme-buffer-water mixtures (size of tube depends on amount of mixtures prepared) (Falcon #2057 or smaller tube)

Spatula

Cutting dish (sterile disposable Petri dish or equivalent)

Sharp scalpel or razor blade for cutting agarose plugs

Bucket of ice or insulated storage box at -20°C

Orbital rocker or equivalent at 25°C

Preparing and Running the Gel:

55-60 $^{\circ}\text{C}$ stationary water bath

Scale for measuring agarose

0.5X TBE (see Buffer Solutions, page 14)

2000 ml graduated cylinder

Sterile, Type I water, prewarmed to 55°C

SeaKem Gold agarose (BMA #50152)

500 ml flask

Microwave

Heat-resistant gloves

Gel leveling table (Bio-Rad #170-4046) or equivalent

1.8% SeaKem Gold agarose (if filling gel wells)

CHEF system for running pulsed-field gels (DR II, DR III, or CHEF Mapper)

Gel casting platform (21 x 14 cm or 14 x 13 cm) and appropriate comb and comb holder

Spatula

KimWipe tissues or equivalent

Staining and Documentation:

Latex (or equivalent) gloves

Ethidium bromide solution, 10 mg/ml (AMRESCO #X328 or equivalent)

(Optional: Destaining bags for disposal of ethidium bromide [AMRESCO E732-25])

Containers to stain and destain gel (22.5 x 22.5 x 5 cm, Nalgene #5705-2020 or equivalent)

Distilled water, 2 liters

Gel Doc 2000 (Bio-Rad) or equivalent gel documentation apparatus



OPN Laboratory Protocol for Molecular Typing of *S. aureus* by PFGE

METHODS: PREPARATION OF PFGE PLUGS

Please read all instructions carefully before starting protocol. Treat all plasticware, glassware, pipettes, spatulas, and other items 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 10% bleach for at least 30 minutes if they will be washed and reused. When possible, all stock buffer and reagent solutions (see Buffer Solutions, pages 13-14) should be prepared in advance (autoclaved or filter-sterilized) and enzyme solutions should be prepared just before use and held on ice until needed.

Step 1: Grow Cultures

Inoculate 1 colony of each test culture from trypticase soy agar plates containing sheep blood into 5 ml Brain Heart Infusion Broth (BHI) in 15 x 100 mm screw-cap tubes. Vortex tube after inoculation. Incubate tube with shaking at 35–37°C for 18-24 hours.

Note: Plugs of standard strain *Salmonella* serotype Branderup strain H9812 from a lot previously made and pretested should be used as standards in each gel. Pretested plugs have previously passed as controls on plug preparation and can therefore be used in each gel as a test for enzyme efficacy.



Step 2: Prepare Plugs

1. Turn on 37°C and 55-60°C stationary water baths. Turn on power to spectrophotometer (if using spectrophotometric standardization of cells).
2. When 55-60°C bath reaches desired temperature, prepare 1.8% SeaKem Gold agarose (BMA #50152) in TE buffer (0.9% is final plug concentration after cell suspension is added) as follows:
 - a. Pour into a clean beaker the approximate amount of stock TE buffer (see Buffer Solutions, page 13) needed for preparing plug agarose and washing plugs. This will be the working TE buffer solution. When finished preparing plug agarose and washing plugs, discard working TE buffer.
 - b. Weigh 1.8 g SeaKem Gold agarose into a 250 ml screw-cap flask.
 - c. Add 100 ml working TE buffer. Swirl gently to disperse agarose.
 - d. Mark the meniscus of the liquid on the flask. Remove screw-cap. Cover flask loosely with clear film. Microwave for 30 - 60 seconds, mix gently, and repeat for 10-second intervals until all agarose is completely dissolved. The time needed to dissolve will depend upon the microwave and power level used.
 - e. Check meniscus, and replace any fluid lost due to boiling off during microwaving with prewarmed, Type I (reagent grade) water. Microwave 15 seconds to homogenize the suspension.
 - f. Recap flask and place in 55°C water bath to equilibrate for 30 minutes. Use lead ring weight to anchor the flask.

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

3. Set up and label the following tubes with culture numbers for each plug to be made:
 - 1.5 ml microcentrifuge tube
 - 17 x 100 mm polystyrene tube (Falcon #2057)
4. Prepare cell suspensions. Adjust concentration of cell suspensions with:



i) MicroScan Turbidity Meter (Dade International, Inc.):
Zero the instrument with sterile BHI. Vortex the cell suspension and measure in the turbidity meter. Adjust turbidity of culture with sterile BHI to turbidity reading 1.1-1.3.

OR

ii) Spectrophotometer:
Set wavelength to 610 nm, and zero the instrument with BHI. Transfer about 1 ml of 18-24 hour broth culture in BHI to micro-cuvette and read absorbance. Adjust absorbance of culture to attain Abs = 0.9-1.1 at 610 nm.

Note: Refer to Biosafety Precautions for disposal of contaminated materials.

Note: The final volume of the cell suspension will depend on the specifications of the spectrophotometer or turbidity meter used to measure the cell concentration. Disposable plastic or glass cuvettes or tubes that fit the instrument can be used for preparing and checking the concentrations of the cell suspensions. Please read the manual for your instrument for the sizes and materials (i.e., plastic or glass) of cuvettes or tubes.

5. Transfer 200 μ l adjusted cell suspension in BHI to microcentrifuge tube. Centrifuge at 13,000 rpm for 3-4 minutes, and aspirate all supernatant. (Note: If supernatant is fully removed, it is not necessary to wash the pellet.) Resuspend pellet in 300 μ l working TE buffer, and equilibrate tubes in 37°C water bath for 10 minutes.
6. Label wells of large plug mold (use strip of masking tape rather than writing directly on the plug mold itself). Small plug molds can be labeled by writing directly on the mold. Remove microcentrifuge tube from water bath, add lysostaphin stock solution directly to cell suspension, and mix gently but quickly (use 3 μ l Recombinant [Sigma #L-0761] or 4 μ l conventional [Sigma #L-7386] lysostaphin per tube. Stock solutions are 1 mg/ml in 20 mM sodium acetate, pH 4.5).
7. Working quickly but carefully so as to avoid shearing the DNA and to avoid creating bubbles in the tube or subsequently in the plug mold, add 300 μ l agarose (equilibrated to 55°C) to the cell suspension, gently mix (use separate Pipetman set to 600 μ l to mix), and dispense mixture into well(s) of either a large plug mold (each well = ~250 μ l) or into well(s) of small disposable mold (~100 μ l each). At least two plugs per specimen are recommended.
8. When all agarose-cell mixtures have been dispensed, allow plugs to solidify at room temperature for 10-15 minutes. They can also be solidified in the refrigerator (4°C) for 5



minutes. Leftover agarose may be stored at room temperature for plug preparation reuse (1-2 times) or to use later in sealing gel wells (see optional step, page 11). For remelting, mark the meniscus, microwave on low-medium power for 10-15 seconds, and mix. Repeat in 5-10 second intervals until agarose is completely melted. Check meniscus, and replace any fluid lost due to boiling off during microwaving with preheated, Type I water. Microwave 15 seconds to homogenize the suspension.

Step 3: Lysis

Using a spatula, remove plug to labeled 17 x 100 mm tube containing at least 3 ml EC lysis buffer (see Buffer Solutions, page 13). Be sure that the plug is fully immersed in the EC lysis buffer and that the water level in the bath is above the level of the EC lysis buffer in the tubes. Incubate at 37°C for at least 4 hours³.

Note: The tape, gel mold, and other items in contact with the organism or plug are contaminated. Discard in appropriate disinfectant or disinfect plug molds in 10% bleach for 30-60 minutes if they will be washed and reused.

³If many plugs are to be prepared, they can be incubated overnight in EC lysis buffer.



Step 4: Rinse Plugs⁴

1. Carefully pour or vacuum off the EC lysis buffer into an appropriate discard container. Plugs can be held in tubes with a spatula.
2. Add at least 4 ml working TE buffer to the 17 x 100 mm tube and agitate (orbital rocker) for 30 minutes at room temperature. Be sure that plug is immersed in TE buffer.
3. Remove buffer and repeat the washing procedure at least three more times.
4. After all washes, add 4 ml of working TE buffer to the 17 x 100 mm tube and store in refrigerator until preparation of enzyme digests. For long-term storage, small plugs can be transferred to smaller tubes (12 x 75 mm or equivalent) with appropriate amounts of TE buffer; large plugs can be stored in the 17 x 100 mm tubes.

⁴The washing with TE buffer to remove the lysis buffer from the plugs can be performed for longer than 30 minutes. The washing can also be started on one day and finished the next day. If DNA of new plugs appears cloudy or hazy but pretested *Salmonella* serotype Branderup strain H9812 plugs produce clear bands, test culture plugs may need additional washing.



OPN Laboratory Protocol for Molecular Typing of *S. aureus* by PFGE

METHODS: PROCEDURE FOR RUNNING PFGE GELS

Step 1: *Sma*I Restriction Enzyme Digestion

1. Calculate total number of restriction digests to be performed (one per culture plus appropriate number of *Salmonella* serotype Branderup strain H9812 standard plug slices [See below]). Label 1.5 ml microcentrifuge tubes.

Number of NCTC 8325 standard plug slices needed per gel

Number of wells per gel	Number of standards per gel	Maximum number of test samples per gel
10 wells	at least 3	7
15 wells	at least 3	12
20 wells	at least 4	16
30 wells*	at least 5*	23*

*Do not use 1st or 30th well for standards or test samples.

2. Prepare sufficient water-buffer mixture (10X Buffer stock diluted 1:10 with sterile, Type I water) in sterile plastic tube (Falcon #2057 or smaller tube) to dispense 200 μ l per microcentrifuge tube (see table below). Mix and dispense into labeled microcentrifuge



tubes.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices	µl/20 Plug Slices	µl/28 Plug Slices
Sterile, Reagent Grade Water	180 µl	1800 µl (1.8 ml)	2700 µl (2.7 ml)	3600 µl (3.6 ml)	5040 µl (5.04 ml)
Buffer (10X stock)	20 µl	200 µl	300 µl	400 µl	560 µl
Total Volume (200 µl x # plug slices)	200 µl	2000 µl (2.0 ml)	3000 µl (3.0 ml)	4000 µl (4.0 ml)	5600 µl (5.6 ml)



3. Cut plug portion.
 - a. Using a spatula, remove plug from storage tube and place in cutting dish (e.g., sterile, disposable Petri dish).
 - b. Trim the uneven edge of plug using a sharp scalpel or razor blade. Cut slice of plug to desired comb size (2 x 5 mm for 20- or 30-tooth comb or 2 x 10 mm for 10- or 15-tooth comb). Cut appropriate number of plug slices of *Salmonella* serotype Branderup strain H9812 standard for gel size and number of test samples (see first table on page 8).
 - c. Place plug slice in labeled microcentrifuge buffer tube and equilibrate in water-buffer mixture at room temperature for 30-45 minutes. Be sure plug slice is under water-buffer mixture.

4. While plug slices are equilibrating in the water-buffer mixture, prepare sufficient enzyme-water-buffer mixture (10X Buffer stock diluted 1:10 with sterile, Type I water and *SmaI* enzyme) to dispense 200 µl per microcentrifuge tube (see table below). The mixture can be prepared in the same tube used for preparation of the water-buffer mixture. Mix gently, making sure enzyme is evenly distributed.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices	µl/20 Plug Slices	µl/ 28 Plug Slices
Sterile, Reagent Grade Water	177 µl	1770 µl (1.77 ml)	2655 µl (2.66 ml)	3540 µl (3.54 ml)	4956 µl (4.96 ml)
Buffer (10X stock)	20 µl	200 µl	300 µl	400 µl	560 µl
<i>SmaI</i> Enzyme (need 30 units/tube)	3.0 µl*	30 µl*	45 µl*	60 µl*	84 µl*
Total Volume (200 µl x # plug slices)	200 µl	2000 µl (2.0 ml)	3000 µl (3.0 ml)	4000 µl (4.0 ml)	5600 µl (5.6 ml)

* *SmaI* stock concentration is 10 units/µl.

Note: Keep enzyme on ice or in insulated storage box at -20°C while in use and use only one lot of enzyme per gel.



Note: If *SmaI* enzyme stock concentration is not 10 units/ μ l:

a. Calculate amount of enzyme needed:

30 units (stock concentration of *SmaI* in units/ μ l) = μ l of *SmaI* needed per plug slice

b. Calculate how much total *SmaI* is needed:

(μ l of *SmaI* needed per plug slice) x (number of plug slices) = total *SmaI* needed

c. Calculate how much sterile, reagent grade water is needed:

Add total *SmaI* + 10X buffer (1:10 of Total Volume). Subtract amount from Total Volume [calculated by 200 μ l x number of plug slices to digest].

5. After plug slices have equilibrated in water-buffer mixture for 30 minutes, remove buffer from plug slice by placing a pipet fitted with a 200-250 μ l tip all the way to the bottom of the tube and aspirating buffer. Do not cut plug slice with pipet tip. Be sure plug slice is not discarded with pipet tip.
6. Add 200 μ l water-buffer-enzyme mixture (prepared in #4, page 9) to each tube. Close tube and mix by gently tapping on tube. Be sure plug slices are under enzyme mixture.
7. Incubate tubes with *SmaI* at 25°C and *XbaI* at 37°C for at least 3 hours⁵. Be sure plug slices are under enzyme mixture. Turn on 55-60°C stationary water bath for Step 2: Running the Gel (below).

Step 2: Running the Gel

1. Prepare running buffer (0.5X TBE) (see Buffer Solutions, page 14):

Calculate total amount needed:

2000 ml (if residual buffer was left in tubing after the previous gel use)

OR

2200 ml (if tubing was flushed with water after the previous gel use)

+ 150 ml for "large" gel (21 x 14 cm) or 100 ml for "small" gel (14 x 13 cm)

2. Prepare running gel in 1% SeaKem Gold agarose.

a. **For the 150 ml "large" gel (21 x 14 cm)**, weigh out 1.5 gm agarose in 500 ml flask and add 150 ml 0.5X TBE buffer. Mark the meniscus and microwave to dissolve. Swirl the flask gently but thoroughly to mix, making sure that agarose is completely dissolved.

⁵ 30 minutes needed if using Promega enzymes. This is shortened to 15-30 minutes if using New England Biolabs enzymes. The enzyme digestion step can be set up late in the afternoon and can proceed overnight.



Replace evaporated fluid with prewarmed sterile, Type I water to meniscus mark. Place molten agarose into 55°C water bath. Equilibrate for 15-20 minutes.

OR

b. For 100 ml “small” gel (14 x 13 cm), weigh out 1 gm agarose in 500 ml flask and add 100 ml 0.5X TBE buffer. Mark the meniscus and microwave to dissolve. Swirl the flask gently but thoroughly to mix, making sure that agarose is completely dissolved. Replace evaporated fluid with prewarmed sterile, Type I water to meniscus mark. Place molten agarose into 55°C water bath. Equilibrate for 15-20 minutes.

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

3. As appropriate (see Step 2. #1, this page), pour 2000 ml or 2200 ml running buffer (0.5X TBE) into gel box. Turn pump on and set at 70 for a flow rate of 1 liter/minute. Turn on cooling module (14°C).
4. After enzyme digestion, remove microcentrifuge tubes containing plugs from water bath and line up in order of loading sequence. The following table shows where to place *Salmonella* serotype Branderup strain H9812 organism standards:

Number of wells per gel	Number of standards per gel	Lanes to load standards
10 wells	at least 3	1, 5, 10
15 wells	at least 3	1, 8, 15
20 wells	at least 4	1, 7, 14, 20
30 wells*	at least 5*	2, 9, 16, 23, 29*

*Do not use 1st or 30th well for standards or test samples.

Note: If loading more standards than required, load one standard in the first lane and another standard in the last lane, distributing the other standards in between.

5. Confirm that gel casting platform (gel frame) is on level surface or is level on a gel leveling table. Adjust comb height on comb holder so that comb teeth touch the gel platform. Place comb holder (with comb attached) flat on work surface so that comb side is closest to work surface.
6. To load, pick up each plug slice with spatula, carefully blot excess fluid from plug slice with



tissue, and place plug slice directly on the very end of comb tooth. When all slices have been placed on the comb, carefully lift the comb and “set” it into the comb holder of the gel form. Plastic comb teeth on comb holder should be next to top of gel and plugs should be “under” black comb holder. Push comb to back of comb holder. Be sure all slices are lined up at end of comb teeth and that they touch the black platform evenly.

7. Pour equilibrated agarose carefully into the gel casting platform so as to not dislodge the gel slices. Allow gel to solidify (45 minutes - 1 hour). Carefully remove comb by lifting straight up.

OPTIONAL STEP: While gel is solidifying, remelt agarose left over from plug preparation (see page 6) by microwaving on low-medium power for 10-15 seconds, mixing, and then repeating the microwaving in 5-10 second intervals until agarose is completely melted. Equilibrate in water bath to 55⁰C. When gel has hardened, remove comb and fill the wells with a small amount of remelted agarose. This seals the plugs in place and strengthens the wells when moving the gel for photography.

8. Remove gel from casting platform (gel frame) but leave gel on removable black plate. Wipe off excess agarose from bottom and sides of black plate with tissue. Keep gel on black plate and carefully place gel inside gel frame in electrophoresis chamber. Close cover of chamber.

Note: The 0.5X TBE running buffer should completely cover the gel. Ideally, the buffer should cover to a height ~2 mm above the gel.

9. Set run parameters (for CHEF DR-II, DR-III, and CHEF Mapper) as follows:
Volts = 200 (6v/cm)
Temp = 14°C
Initial switch = 5 seconds
Final switch = 40 seconds
Time = 21 hours for SeaKem Gold agarose

Step 3: Staining and Documentation

1. When run is completed, turn off equipment (turn off chiller before pump). Remove gel from chamber and stain gel with ethidium bromide solution (AMRESCO X328, 10 mg/ml stock; use 50 µl of stock in 500 ml distilled water) for 20-30 minutes in a covered container (e.g., 22.5 cm x 22.5 cm x 5 cm, Nalgene #5705-2020).



Note: Ethidium bromide is toxic and a mutagen. In addition to a laboratory coat, wear latex (or equivalent) gloves when working with ethidium bromide. The solution can be kept in a dark bottle and reused 5-6 times before discarding according to your institution's guidelines for hazardous waste. One method for discarding ethidium bromide is the use of destaining bags (AMRESCO E732-25).

2. Destain in fresh distilled water for 45-60 minutes.
3. While staining and destaining, drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 liters of distilled water. Use pump to drain chamber as thoroughly as possible. Aspirate remaining buffer from chamber. If unit is not going to be used for several days, flush lines with water by allowing pump to run for 5-10 minutes before draining water from chamber.

Caution: Do not let used buffer stand in gel chamber overnight or between uses.

4. Place destained gel on UV box, and document using Gel Doc 2000 (Bio-Rad) or equivalent. If both a Gel Doc and conventional photograph are needed, photograph gel first and then capture image on Gel Doc 2000 or equivalent.



OPN Laboratory Protocol for Molecular Typing of *S. aureus* by PFGE

BUFFER SOLUTIONS

TE BUFFER*:

Used in all PFGE procedures for preparing agarose used in making plugs, for washing plugs after lysis, and for storage of washed plugs.

Final concentration: 10 mM Tris HCl, 1 mM EDTA, pH 8

Use Stock solutions:

	<u>For 2000 ml</u>
1M Tris HCl, pH 8 (Gibco/BRL #15568-017)	20 ml
0.5M EDTA, pH 8 (AMRESCO #E177)	4 ml

QS to 2000 ml with Type I (reagent grade) water in graduated cylinder. Mix thoroughly.

Transfer to two 1000 ml glass screw-cap bottles (Wheaton #219760).

Loosely cap, and autoclave on liquid cycle for 20 minutes.

Store at room temperature for up to 6 months.

If sterile water, stock solutions, and glassware are used, no autoclaving is necessary.

EC LYSIS BUFFER*:

Final concentration: 6 mM Tris Hcl
1 M NaCl
100 mM EDTA
0.5% Brij-58
0.2% Sodium deoxycholate
0.5% Sodium lauroylsarcosine

Use stock solutions or powders:

	<u>For 1800 ml</u>
1M Tris HCl, pH 8 (Gibco/BRL #15568-017)	10.8 ml
5M NaCl (AMRESCO #E529)	360 ml
0.5 M EDTA, pH 8 (AMRESCO #E177)	360 ml
Brij-58 (Polyoxyethylene 20 Cetyl Ether; Sigma #P-5884)	9 gm
Sodium deoxycholate (Sigma #D6750)	3.6 gm
Sodium lauroyl sarcosine (Sigma #L5125)	9 gm
Type I water	~700 ml

Add solutions and powders to beaker, use stir bar to mix thoroughly. Heat on low heat to



facilitate complete dissolving. Pour solution carefully (to minimize foaming) into 2000 ml graduated cylinder, QS to 1800 ml with Type I water. Pour back and forth between cylinder and beaker to mix thoroughly. Autoclave solution in sterile, glass screw-capped bottles. Store at room temperature for up to 6 months.

Tris-Borate EDTA Buffer (TBE)*:

Preparing 0.5X TBE from 5X TBE (AMRESCO #J-885)

Reagent	Volume in milliliters (ml)					
5X TBE	200	210	220	230	240	250
Reagent Grade Water	1800	1890	1980	2070	2160	2250
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

Preparing 0.5X TBE from 10X TBE (Gibco/BRL #15581044)

Reagent	Volume in milliliters (ml)					
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

***Troubleshooting:**

- To keep from contaminating stock buffers, dispense approximate amounts of stock buffer needed into a separate, sterile beaker as a “working buffer”. When finished with working buffer, discard. Do not pour working buffer back into stock buffer.



- TBE buffer contamination generally cannot be detected by visual inspection. If you are having trouble with enzyme digests, one component to suspect is contaminated buffer. Discard old buffer and make new buffer.



ESP Buffer

1. To prepare 0.5 M EDTA, pH 9.2-9.5

For 1 liter:

186.1 gm Na₂EDTA 2H₂O
600 mL R/O or distilled water
70mL 10M NaOH

For 2 liters:

372.2 gm Na₂EDTA 2H₂O
1200 mL R/O or distilled water
140mL 10M NaOH

*Adjust pH \geq 8.0 with 10M NaOH because EDTA won't readily go into solution at less than pH 8.

*Turn heat on low and stir to dissolve completely. Turn off heat and allow solution to return to room temperature.

*Add additional 10M NaOH with stirring. Let pH "steady out" each time before adding more NaOH. Final pH should be 9.2—9.5. For one liter it takes about 20 to 23 mL additional NaOH.

*QS to 1000 mL in a 1000 mL graduated cylinder. Pour back and forth to mix thoroughly. Then proceed to step 2.

*If making 2000 mL, QS to 2000 mL. Then filter sterilize 1000 mL of the solution using a 0.2 μ disposable filter unit. Transfer to a sterile Vitro bottle for use later. Use the remaining 1000 mL for step 2.

2. Add 10 gm of Sodium laurylsarcosine to 1000 mL of the above solution. Stir to dissolve. Heat with low heat if necessary to dissolve. Filter sterilize using a 0.2 μ filter unit. Transfer to a Vitro bottle for storage.



GRAM-NEGATIVE LYSIS BUFFER

Final concentration: 1M NaCl

100 mM EDTA (pH 8)

0.5% Sarkosyl

0.2% Sodium Deoxycholate

Use Stock solutions or Powders:

For 1800 ml (4000 ml beaker)

5M NaCl (AMRESCO)

360 ml

0.5M EDTA, pH 8 (AMRESCO)

360 ml

Sodium laurylsarcosine (Sigma)

9.0 gm

Sodium deoxycholate (Sigma)

3.6 gm

RO water

~700 ml

Add solutions and powders to beaker, add stir bar to mix thoroughly. Heat on low heat to facilitate complete dissolving.

Pour solution into 2000 ml graduated cylinder, QS to 1800 ml with RO water.

Pour back and forth between cylinder and beaker to mix thoroughly.

Filter sterilize using 0.22 μ disposable filter unit (500 ml at a time), transfer to two sterile 1000 ml Vitro (Wheaton) bottles, ~900 ml/bottle

For Use

Determine total amount buffer needed for all plugs

i.e., for 27 plugs, need

Weigh out (analytical balance) lysozyme (Sigma)

$27 \times 2.5 = 62.5$ ml

(Need 1 mg lysozyme/ml lysis buffer, rounded down if decimal amount)

plus 0.5 ml extra = 63 ml total

i.e., for 27 plugs, use 63 mg lysozyme

for 30 plugs, need

for 30 plugs, use 75 mg lysozyme

$30 \times 2.5 + 0.5 = 75.5$ ml total

Transfer lysozyme powder to 125 or 250 ml

screw-capped flask; measure out needed buffer

in 100 or 250 ml graduated cylinder, add to flask

Equilibrate lysis buffer-lysozyme flask at 37°C for

20-30 min after adding lysozyme



Short Procedure for PFGE of Gram negative rods (including Salmonella serotype Branderup strain H9812)

Step 1: Grow culture

Inoculate 25 mL of BHI broth containing 0.5% beef extract with one colony from a 16-18 hour growth of Gram negative rod from a Blood Agar plate. Incubate broth culture at 35-37 degrees C on a shaker for about 16-18 hours. If the organism grows better at a lower temperature (e.g. 25°C or 30°C) incubate on a shaker at the preferred temperature.

Step 2: Prepare plugs

1. Prepare 1.8% agarose (e.g. Seakem Gold) using TE buffer. Microwave to dissolve and place in 65 degree C waterbath until ready to use. Allow 300 µL agarose for each plug.
2. Label 1.5 mL microfuge tubes, one for each cell suspension.
3. Transfer 200 to 250 µL of the cell suspension (depending on turbidity) to a microfuge tube.
4. Centrifuge at 12,000 rpm for 4 to 5 minutes.
5. Aspirate the supernatant leaving the pellet intact.
6. Add 300 µL of TE Buffer to each pellet and vortex to disperse the pellet.
7. Place the microfuge tubes in a 37 degree C waterbath for at least 15 minutes.
8. Prepare the lysozyme/Gram negative lysis buffer. Allow 3 ml of this mixture for each plug. Prepare by adding 1 mg of lysozyme for each ml of gram negative lysis buffer. Place into a 37 degree C waterbath until needed.
9. Assemble the plug mold.
10. Remove microfuge tubes from the waterbath. Vortex the tubes briefly before adding 300µL of the 1.8% agarose. Mix with your pipette tip, and then transfer the mixture into the plug mold. Place the mold on ice or in the refrigerator for 15-20 minutes.
11. Add 3 mL of the lysozyme/gram negative lysis buffer solution into sterile tubes. (17 x 100 mm tubes with caps work well).
12. Using a teflon spatula, remove an agar plug from a plug mold and transfer to a tube containing 3 mL of the lysozyme/gram negative lysis buffer solution.
13. Place these tubes in a 37 degree waterbath overnight or for at least 4 hours.
14. Prepare an ESP/proteinase K solution. Allow 1 mg Proteinase K per mL of ESP. Prepare 2.5 mL of the solution for each plug. Place the solution in a 50 -55 degree C waterbath until needed.
15. After the 37 degree C incubation, remove the tubes and drain off the lysozyme/gram negative buffer solution.
16. Add 2.5 mL of the ESP/Proteinase K to each tube and incubate at 50 -55 degrees C overnight.



17. Remove tubes from waterbath, drain off ESP/Proteinase K solution and wash at least 3 times with TE buffer. Wash with 4 mL of TE buffer and rotate gently for 30 minutes each time. Discard last wash and replace with 3 ml of TE buffer. Store at 4 degrees C until ready to use.

Running the gel: Step 3

1. Prepare 2000 ml of 0.5% TBE buffer.
2. Prepare a gel by adding 1.5 grams of Agarose (e.g. Seakem Gold) to 150 mL of the 0.5% TBE buffer. Microwave to dissolve the agarose and place the mixture at 50-55 degrees C until needed.
3. Cut a thin slice of the agarose plug to be tested. For a 20 or 30 tooth comb the slice to load should be about 2 mm x 4.5 mm x 1.2 mm.
4. Place the slice into a 1.5 mL microfuge tube containing 125 μ L of the appropriate buffer. Add the appropriate amount of restriction enzyme and bovine serum albumin

Incubate at 37°C for 4 hours or overnight. (15 – 30 minutes if using New England Biolabs enzymes)

5. After incubation in buffer/enzyme solution, place the slices evenly on the teeth of the comb. Include the Universal Standard (Salmonella braenderup digested with 3 μ L Xba I in 125 mL of the appropriate buffer plus 2 μ L of bovine serum albumin) to each run.
6. Place the comb into the gel mold. Slowly add the 150 mL of the agarose prepared in step 2 to the gel mold. Allow the agarose to solidify for at least 30 minutes.
7. Meanwhile place the remaining 0.5% TBE buffer from step 1 (about 1850 mL) into the Chef rig. Set the cooling temperature at 14 degrees C.
8. Remove the comb and place the solidified gel into the gel box.
9. Set the appropriate switch times and the gel run time for the organism being tested.
10. Start run.

Staining the gel:

1. Remove the gel from the gel box.
2. Stain for 15 minutes in a 5% EtBr solution (i.e. 25 μ L ethidium bromide and 500 mL R/O or distilled water).
3. Rinse for 45 minutes to 1 hour in a liter of R/O or distilled water.
4. Photograph using UV light.



SHORT PROTOCOL FOR *STAPHYLOCOCCUS AUREUS* PFGE

1. Inoculate a single colony of *Staphylococcus aureus* into BHI broth and streak a BAP with the same colony. Incubate cultures overnight at 35-37°C.
2. Prepare 1.8% SeaKem Gold agarose in TE buffer. Place in 65°C waterbath.
3. Adjust BHI broth suspension to a MicroScan reading of 1.1 to 1.3.
4. Transfer 200 uL of the cell suspension to a microcentrifuge tube.
5. Centrifuge at 13,000 for 3-5 minutes.
6. Aspirate supernatant.
7. Resuspend pellet in 300 uL TE buffer and equilibrate tubes in 37°C waterbath for at least 15 minutes.
8. Add 4 uL lysostaphin to cell suspension. Vortex.
9. Add 300 uL of the 1.8% agarose to the suspension, and mix with pipette tip.
10. Dispense into plug mold.
11. Refrigerate for 10 minutes.
12. Remove from plug mold and place into a tube containing 3 ml EC lysis buffer.
13. Incubate at 37°C for 4 hours or overnight.
14. Pour off buffer, and replace with 4 ml TE buffer. Place on rotator for 30 minutes.
15. Wash the plug 3 more times with TE buffer.
16. Store the plug at 4°C in TE buffer.

Sma restriction and running PFGE gels

1. Cut *Staph aureus* plug according to instructions and place the fragment in 150 µL of appropriate buffer (Buffer J = Promega; NEB 4 = New England Biolabs)
2. Add 3 uL *Sma* I to plug fragment.
3. Incubate at RT for 4 hours or overnight if using Promega enzyme, 15 – 30 minutes if using New England Biolabs enzyme.
4. Cut plug for *Salmonella braenderup* Standard and place fragment in 150 µL of appropriate buffer (Buffer D = Promega; NEB 2 = New England Biolabs).
5. Add 3µL *Xba*I + 2 µL bovine serum albumin. Place in 37°C waterbath for 4 hours or overnight if using Promega enzyme, 15 – 30 minutes if using New England Biolabs enzyme.
6. Prepare 1% SeaKem Gold agarose for the gel.
 - a. 100 mL TBE Buffer + 1900 mL R/O water.
 - b. Weight out 1.5 g SeaKem Gold Agarose and place in a 500 mL Erlenmeyer flask.
 - c. Add 150 mL of the TBE Buffer solution.
 - d. Microwave to dissolve agarose.



- e. Place in 55°C water bath until ready to pour gel.
 7. Load comb with Staph plug fragments. Include Salmonella Standards on the first, middle and last places on the comb.
 8. Let fragments settle for 5 minutes then add a single drop of agarose on top of each fragment.
 9. Let fragments settle for an additional 5 minutes
 10. Pour gel. Allow to solidify for 30 minutes.
 11. Run on CHEF system at 200 Volts with the following parameters:
 - Temp 14 degrees C
 - Initial switch time 5 seconds
 - Final switch time 40 seconds
 - Time: 21 hours
 12. Stain with ethidium bromide (a carcinogen) for 15 minutes.
 13. Destain for 45 minutes.
- Photograph using UV light