	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*

Title: Molecular subtyping of *Salmonella, E. coli* O157:H7, *Shigella sonnei, Shigella flexneri* by Pulsed-field Gel Electrophoresis (PFGE)			
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Molecular Subtyping of *Salmonella*, *E. coli* O157:H7, *Shigella sonnei, Shigella flexneri* by Pulsed-field Gel Electrophoresis (PFGE)

FILE NAME: 12JUL16_Salm_PFGE_SOP

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SECTION 1 INTRODUCTION

1.1 Purpose

This protocol describes the standardized method of molecular typing by PFGE of *Salmonella*, *E. coli* O157:H7, *Shigella sonnei*, and *Shigella flexneri* as to be used in the FSL laboratory. This SOP is based on the standardized CDC PulseNet protocol (original PulseNet protocol can be found under the reference section of this SOP).

1.2 Scope

This procedure is used for molecular typing by PFGE of *Salmonella*, *E. coli* O157:H7, and *Shigella* species under the supervision of Martin Wiedmann within the department of Food Science at Cornell University and applicable to all members of FSL, MQIP, and LMT who perform these duties.

1.3 Definitions/Acronyms

PFGE: Pulsed Field Gel Electrophoresis
BHI: Brain Heart Infusion Agar
CSB: Cell Suspension Buffer
CLB: Cell Lysis Buffer
SKG: SeaKem Gold agarose
PK: Proteinase K
EDTA: Ethylenediaminetetraacetic Acid
TBE: Tris Base EDTA
TE: Tris-HCl EDTA
RPM: Revolutions Per Minute
ddH2O: distilled/deionized H2O
RCT: Red-capped tubes
GCT: Green-capped tubes
EtBr: Ethidium Bromide

1.4 Safety

Organisms used with this SOP are all capable of causing human illness and are BSL-2 pathogens. Appropriate protective measures need to be taken when working with *Salmonella*, *E. coli*, and *Shigella*. All waste from experiments involving these organisms needs to be treated as BSL-2 waste. Ethidium bromide is a carcinogen and all waste must be appropriately treated and handled. Appropriate PPE must be worn at all times.

SECTION 2 MATERIALS

Reagents
Note: All solutions prepared in the Wiedmann lab must be less than 6 months old.
1M Tris HCl, pH 8.0
0.5M EDTA, pH 8.0
TE Buffer, pH 8.0
Cell Suspension Buffer (CSB)
Cell Lysis Buffer (CLB)
10X TBE, pH 8.0
0.5X TBE
ddH2O (autoclaved)
dH2O
Lyophilized Proteinase K (Roche Applied Science)
20% Sodium Dodecyl Sulfate (SDS) solution
20 mg/mL Bovine Serum Albumin (BSA) (New England Biolabs)
Restriction Buffer H (Roche Applied Sciences) – buffer for *Xba*I
Restriction Enzyme *Xba*I (10U/µL, Roche Applied Sciences) **(For *Salmonella* H9812 digestion)**
SKG Agarose for PulseNet Lab Use (LONZA)
Ethidium Bromide (1mg/mL)
Thiourea (10mg/mL)

Materials
BHI agar plates
Loops
Sterile wooden sticks
Red-capped tubes (RCT), Borosilicate Tubes 13x100 mm
Green-capped tubes (GCT), Borosilicate Tubes 16x125 mm
Sterile cotton swabs
Micropipettes and tips. Volumes: 20 µL, 200 µL, and 1000 µL
Weighing dishes
Razor
Spatula
Sterile 125 mL bottles
Sterile 1.5 mL Eppendorf tubes
Sterile 15 mL conical tube
Sterile 50 mL conical tube
Plug Mold (BioRad)
Screened caps (BioRad)
Microscope slides
1000 mL graduated cylinder
100 mL graduated cylinder
2000 mL Erlenmeyer flask

Gel mold, frame, and comb (BioRad)
Level tool
70% Ethanol

Equipment
Spectrophotometer
Analytical balance
Water baths (37 °C, 55–60 °C, and 25 °C)
Incubator (37°C)
Shaking incubator (50/54 °C)
Pulsed-field electrophoresis chamber
Cooling module
Control module
Drive module
GelDoc Imaging System, BioRAD

SECTION 3 PROCEDURES

Overview of Typical Work Flow:

Day 1: Streak the isolates on BHI agar and incubate 16–18 h at 37 °C.
Day 2: Plug Preparation & Washing
Day 3: Restriction Digestion (Day 3 and 4 procedures can be finished in 1day)
Day 4: Gel Run
Day 5: Gel Staining & Photographing Gel Image

3.1. Streak isolates for overnight growth:

***Note:** Before working with BSL-2 organisms please review proper handling techniques.

1. Using a sterile wooden stick, streak out isolate(s) from glycerol frozen stock (or a client slant or a single colony from a client plate) onto a BHI plate. Streak bacterial isolates in a zigzag horizontal pattern until 1/3 of the plate is covered (lawn).
   a. Rotate the plate about 90 degrees, and using a sterile loop start spreading the bacteria, at the end of the lawn, in a zigzag horizontal pattern. Repeat this step one more time. This procedure will result in isolated colonies.
2. Allow bacteria to grow in 37 °C incubator overnight (16–18 hours).
   *Note: Always use freshly streaked plates for preparation of cell suspensions as cells are easier to lyse. Overnight growth should be no longer than 18 hours.
3. Streak *S.* Braenderup (FSL W3-083) to make reference plugs. Reference plugs are made in the same manner all other *Salmonella* plugs are made (see below).

3.2. Plug Preparation:

1. Turn on both water baths, the 37 °C and 55–60 °C.
2. Turn on the 54 °C incubator shaker.
3. Follow the spectrophotometer instructions on Appendix 1.
4. Briefly, turn on spectrophotometer, set wavelength to 610 nm, and allow to warm up for ~15 minutes.
5. For each isolate: label a RCT (these will be used to prepare cell suspensions), a 1.5 mL Eppendorf tube, and a 50 mL conical tube.
6. Fill RCTs with 3.5 mL **Cell Suspension Buffer (CSB)**. Fill twice as many GCTs with 2.0 mL **CSB** (these will be used for measuring ODs and

altering concentration of the cell suspensions)

7. Blank spectrophotometer with 3 mL of **CSB** in a GCT.
8. Using a sterile cotton swab that has been pre-moistened with **CSB**, remove cells from the lawn on the BHI plate and suspend the cells in the **CSB** within the RCT by gently swirling sterile swab.
9. Gently, pipette up and down the cell suspension to achieve a homogeneous suspension of cells.
10. Transfer 1.0 mL of this suspension into a GCT containing 2.0 mL **CSB**, and pipette up and down again.
11. Measure the OD_{610} of the GCT cell suspension. Target OD_{610} range is 0.400–0.500.
12. After achieving an OD_{610} within the target range, place the RCT cell suspensions on ice. Repeat steps 8–12 for all isolates.
    *Note: If the OD_{610} is not within the target range, adjust it by adding more bacterial colonies or diluting it with **CSB**.
13. Prepare 1.0% SKG agarose in sterile bottle or flask:
    0.30 g SKG + 23.5 mL TE Buffer → Microwave until completely dissolved and thickened.
    *Note: Agarose solution has to be microwaved until all the agarose is dissolved and thickened (about 1–2 minutes, stopping to swirl several times). Failure to do so will result in plugs that could easily break.
14. Place the agarose solution in the 55–60 °C water bath for at least 10 minutes, but use as soon as possible after equilibrated to 55-60 °C.
15. Distribute 400 µL of adjusted cell suspensions into labeled 1.5 mL Eppendorf tubes and incubate in 37 °C water bath for 10 minutes.
16. Prepare 20 mg/mL Proteinase K solution (to be used in plug prep and in lysing solution) and store on ice:

Proteinase K solution	Amount of plugs
0.013 g PK + 650 µL dd H_2O	~12–14 plugs
0.020 g PK + 1000 µL dd H_2O	~20–21 plugs

17. Add 1.25mL of 20% SDS solution to the agarose, swirl the bottle to make sure they are mixed.
18. Remove cell suspensions from 37 °C water bath and add 20 µL of the PK solution
19. Mix 400 µL of the SKG agarose/SDS solution with the 400 µL cell suspension/PK solution and mix by gently pipetting up and down (no more than 3–4 times to avoid agarose solidification) and immediately cast into cleaned plug mold. Overfill slightly – the plugs shrink as they solidify.
    *Note: Try to avoid bubble formation.
20. Allow the plugs to solidify for 15 minutes.

21. Prepare lysing solution master mix in sterile bottle:
n= # of isolates

5mL CLB x (n+1) =	
25µL PK x (n+1) =	

22. Distribute 5 mL of lysing solution master mix into each 50 mL conical tube.
23. Trim off excess agarose with a razor blade cleaned with 70% EtOH. Open the plug molds carefully, and using a cleaned spatula transfer solidified plugs into corresponding conical tube.
*Spatula can be wiped with a Kimwipe or 70 % EtOH in between plugs.
24. Lyse the bacterial cells in the plugs, within conical tubes, in shaker incubator at 54 °C/ 170 RPM for 3.5 hours.
25. Spray plug mold(s) with 70% EtOH to kill residual bacteria. Wait 10 minutes, then wipe and dry. Rinse well with dH_20 and dry. Rinse well with 70% EtOH and dry. Wipe once more with 70% EtOH and loosely reassemble plug mold. Air dry, then tighten (must be dry) so it's ready for next time.

3.3. Plug Washing:

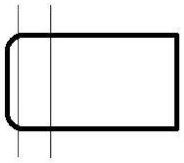
1. Before the washes, pre-warm the ddH_2O and the TE buffer at 50 °C in the water bath.
2. After cells have been lysed, remove conical lid (keep) and replace with green screen caps.
3. Drain lysing solution from plugs and replace with 10 mL autoclaved ddH_2O. Return plugs to shaker incubator for 15 (12-30 okay) minutes at 50 °C/ 70 RPM (change the settings on the shaking incubator).
4. Drain and repeat one time (Total of 2 washes with ddH_2O)
5. Drain the ddH_2O and add 10 mL TE buffer. Return to shaker incubator for 15 (12-30 okay) minutes at 50 °C/ 70 RPM.
6. *Note: You can stop the washes with TE and store the plugs at 4°C, then resume the washes the next day (no more than 24 hrs between washing steps). When you resume the washes, first replace the TE Buffer, then do the first wash for 30 minutes instead of 15. Drain and repeat three more times (Total of 4 washes with TE buffer).
7. Drain the remaining buffer, remove the screen caps, add 5 mL TE, and put lid on.
8. Store the tubes at 4 °C until proceeding to next step of protocol.
9. Thoroughly spray both sides of screen caps with 70% EtOH, air dry, then autoclave (once you've accumulated a container-full) before reusing.
*Note: Plugs stored properly can be digested and run after up to two years.

3.4. Restriction Digest of *Salmonella* Agarose Slices, including the *Salmonella* Reference slices (*Xba*I):

1. Label one 1.5 mL Eppendorf tubes per isolate.
2. Label two appropriately sized tubes (2 mL Eppendorf or 15 mL conical tube) for **H buffer** solution and ***Xba*I enzyme** solution.
3. Prepare **H buffer** solution:

H buffer solution	Amount/ plug slice	x (n+1)	Amount in Master mix:
Autoclaved dd H_2O	180 µL		
Restriction Buffer H	20 µL		

4. Distribute 200 µL of restriction buffer solution into each Eppendorf tube.
5. Using cleaned razor and spatula, cut rounded bottom edge of plug off and discard. Cut a ~3 mm slice from the plug (as depicted below) and place it in H buffer solution. Do not cut slices smaller than 2mm.



*Note: The shape of your plug slice will determine the shape of your resulting pattern, so it is important to cut a clean sharp rectangle. (Spatula and razor can be wiped with a Kimwipe or cleaned with 70% EtOH in between plugs.)

6. Incubate Eppendorf tubes (with H buffer solution + slices) in 37 °C water bath for 10 minutes.
7. Prepare restriction enzyme solution:

***Xba*I solution**	Amount/ plug slice	x (n+1)	Amount in Master mix:
Autoclaved dd H_2O	174 µL		
Restriction Buffer H	20 µL		
Restriction Enzyme *Xba*I (10U/µL)	5 µL		
BSA	1 µL		

8. Take out the tubes from the water bath, and dry their external surface. With pipettor tip, aspirate off buffer solution from plug slices and replace with 200 µL *Xba*I enzyme solution.
9. Return slices to the 37 °C water bath, and incubate for 4 hrs (3-5.5 is OK).

10. After digestion, aspirate off enzyme solution in the Eppendorf tubes, replace it with 200 µL TE Buffer and store at 4 °C until next step of the *Note: You can digest your reference plugs ahead of time. A digested plug could be stored at 4 °C for up to two weeks.

3.5. Gel preparation and gel run:

***Note: Please refer to Appendix 2 for screen shots of the Equipment and front panels.**

1. Turn on 55–60 °C water bath.
2. Before the gel is cast, the pulsed-field electrophoresis chamber needs to be prepared.
   a. Make sure all of the tubing is connected so the buffer will circulate through the cooling module. Secure the appropriately sized frame in the chamber.
   b. Place the level tool in the center of the chamber. If it is not leveled, adjust the corner feet of the chamber accordingly.
3. Prepare the running buffer and pour it into the pulsed-field electrophoresis chamber.

Reagents	Volumes
dH_2O	2090 mL
10X TBE	110 mL

*Note: After diluting the 10X TBE its concentration changes to 0.5X

4. Turn on the system (CHEF-Mapper or CHEF-DRII) in the following order and make sure that the pump is set at 70 RPM (Fig. 1):

Steps	CHEF-Mapper (Location of button)	CHEF-DRII (Location of button)
1	Control Module (front)	Drive Module (back)
2	Pump (front of Control Module)	Control Module (right side)
3	Cooling Module (front)	Pump (front of Drive Module)
4	-	Cooling Module (front)

5. The Cooling module temperature should be set at 14 °C, and the "Actual Temperature" button (on the front of the Cooling module) should be pushed (Fig. 2).
6. Prepare 1% SKG gel in sterile flask:

Reagents	10-well gel (small gel)	15-well gel (large gel)
SKG agarose	1.0 g	1.5 g
10X TBE	5.0 mL	7.5 mL
d H_2O	95.0 mL	142.5 mL

7. To avoid additional water loss in the microwave process, weigh gel flask (containing agarose, and 10X TBE diluted in dH_2O) and zero scale. Add 8 mL autoclaved d H_2O and microwave. Microwave gel flask until these additional 8.0 mL of water has evaporated and scale returns to zero.
8. Put the flask in the 55–60 °C water bath for 10 minutes.
   *Optional: Take the flask out of the water bath and let it stand on the bench for 5 minutes.
9. Assemble and clean the gel mold with 70% EtOH. Position sides so the grooves face inward and use them to secure the gel tray. Position the comb so the teeth are closest to the top edge of the gel. Make sure the gel mold is level, using the level tool. If it is not level, move the mold over a bench where it is level or add paper towels under the edge/corner to level. Cover the gel mold until the gel is poured – to keep dust out.
10. Pipet 1.5–2.0 mL of gel for sealing the wells into an Eppendorf tube. Keep extra gel (in the tube) from solidifying by placing in 55–60 °C water bath. Pour gel into cleaned gel mold. Make sure comb is centered and straight.
    *Note: Do not let gel stand for a long time before pouring as this will result in a "swirly" effect in the solidified gel.
11. Cover gel and gel mold with tupperware container to avoid dust falling into the mold. Position cover to allow steam to escape.
12. Allow gel to solidify for 20–25 minutes.
13. Remove digested plug slices from 4 °C and aspirate off TE Buffer. Replace with 200 µL of 0.5X TBE.
14. Using a cleaned spatula, remove slices from Eppendorf tubes and blot slice gently with Kimwipe to remove excess of 0.5X TBE Buffer and load into well. *Note: When loading slices it is important to not damage/ crush slices, load them unevenly within the well, or create air bubbles in wells. All these will disrupt the resulting patterns.
15. Push the plugs carefully against the well's wall closest to you, this will help to avoid bubbles.
16. If using 10-well gel, lanes 1 and 10 should be used for reference slices. If using 15-well gel, lanes 1, 8, and 15 should be used for reference slices.
17. When all slices are loaded, seal slices into wells by slowly pipetting saved agarose from step #10 onto slices.
18. Wipe any excess agarose off of the bottom of the gel tray, then put the agarose gel into the frame within the electrophoresis chamber.
19. Add 860 µL 10 mg/mL Thiourea to the running buffer in the electrophoresis chamber. Circulate for at least 2 minutes before starting the run.
20. Set the parameters on the Control module.

Parameters for PFGE (Fig. 3 & 4) (*check for current run times posted on PFGE equipment):

CHEF-DR II

Steps	Instructions	Numerical input
1	Push the "block" button and make sure that it is set to **b-1**.	b-1
2	Push the "Volts/cm" button and set the voltage.	V = 6
3	Simultaneously, push the "Block" button and the "Volts/cm" buttons to set the Initial switch.	Initial Switch = 2.2 s
4	Simultaneously, push the "Volts/cm" button and the "Run Time" buttons to set the Final switch.	Final Switch = 63.8 s
5	Push the "Run Time" button and set the time.	Small 10-well gel = 17.0* hr
		Large 15-well gel = 18.5* hr

CHEF-Mapper

Steps	Instructions	Numerical input
1	Push the "Auto Algorithm" button.	-
2	"Low [ ]" and "High [ ]" will show on the screen of the Control module. You have to type the values as well as the units For Low: -Type 30, and then push the "Kb" button. -Push "enter" button. For High: -Type 700, and then push the "Kb" button. -Push "enter" button.	Low = 30 Kb
		High = 700 Kb
3	Percent (%) of agarose should be set already at 1% -Push "enter" button.	1 % Agarose
4	Gradient should be already set at 6.0 V/cm. -Push "enter" button.	6.0 V/cm
5	"Run Time [ ]" will show on the screen. You have to type the values as well as the units. -Type 18.5, and then push the "hours" button. -Push "enter" button.	Small 10-well gel = 17.0* hr
		Large 15-well gel = 18.5* hr
6	"Included Angle" should be set already at 120 ° -Push "enter" button.	120 °

7	"Initial Switch Time [ ]" and "Final Switch Time [ ]" will show on the screen. You have to type the values as well as the units For Initial Switch Time: -Type 2.16, and then push the "seconds" button. -Push "enter" button. For Final Switch Time: -Type 1.03.08, and then push the "seconds" button. -Push "enter" button.	 Initial Switch = 2.16 s Final Switch = 1.03.08 s
8	Push "Start Run"	

21. After setting up the machine, write down the Current (mA)
    *mA at t=0 should be ~135 +/- 15 mA
22. After starting the run, reset the temperature probe by pressing the "Memory clear" button.
23. Clean all parts of the gel mold right away (or soak in dH_2O overnight and clean the following morning). Rinse well with dH_20 and dry. Rinse well with 70% EtOH and dry.

*Run times must be empirically determined, make sure every three months that the running times are still appropriate. Replace electrodes when necessary.

3.6. Staining and Photographing Gel:

1. Go to the staining room and properly dispose of the Ethidium Bromide in the flask with the destaining tea bags (please see **Appendix 3)**.
2. Prepare the staining solution in a large container. *Note: Make sure you prepare it fresh EVERY time:

700 µL EtBr (1mg/mL)	700 mL d H_2O

3. Fill the destaining container with 2 L of fresh dH_2O (not with tap water).
4. Turn off the Cooling Module, the pump, and Control Module (Appx. 2 Fig. 1)
5. Open the chamber, remove the gel and take it to the staining container.
6. Stain for 40 minutes and destain it for ~1 hour. While staining you MUST clean the pulsed-field electrophoresis chamber as described at the end of this Section, entitled: "Cleaning the PFGE Equipment".

***Note: Please refer to Appendix 4 for screen shots of GelDoc imaging system and QuantityOne software.**

7. Take the gel to the GelDoc imaging system, turn it on (switch on the back of the device). (Fig. 1)
8. Open the drawer and place the gel on the top of it. Close it.
9. Click on the QuantityOne software icon on the computer screen. (Fig. 2)
10. Click on "Basic" on the screen (Fig. 3)
11. Open Live image by selecting File > GelDoc XR.
12. Push the "Epi white" button on the GelDoc system panel (Fig. 1).
13. Open the door of the GelDoc system and center gel while looking at the grid on the computer screen (Figure 4). "Zoom" in/out to fit your gel. If you do not see the grid click on "Live/Focus". It is important to center gel as this will be the final image uploaded into BioNumerics for analysis. Dry the area around the gel with a Kimwipe so the gel stays in place.
14. Click on "Manual Acquire" on the computer screen (Fig. 4)
    A good starting exposure time should be ~0.500.
15. Push the "Trans UV" button on the GelDoc system panel (Fig. 1). The UV light will not turn on unless the drawer and door are both latched shut.
16. If necessary adjust exposure and focus on the computer screen (Fig. 4).
17. Click on "Freeze" on the screen, then push the "Trans UV" button on the GelDoc system panel - it is important to turn off the UV light after taking the picture to avoid overexposure.
18. Double click on "Save" and choose the folder you want to store your picture. If your isolates are from a client, save the picture within the PFGE folder in the server>Salmonella folder >Client's folder. Save it as Net ID+Date+time of the gel, for example " mw16 05-07-2013 12:36pm"
19. Double click on "Print" and paste the copy for your lab notebook (Fig. 4).
20. Once the gel picture has been captured, the file must be exported into a TIFF image file that BioNumerics recognizes.
21. To export into TIFF image, follow these steps: click on File > Export to TIFF image
    a. Under Publishing section click on "Export view excluding overlays"
    b. Under Resolution section click on "Same as Scan"
    c. Then click on "Export"
22. For information regarding processing gel fingerprint and labeling in BioNumerics, refer to SOP entitled "CU Naming SOP".
23. After the image is captured and exported, return the gel into the destaining container and clean the PFGE equipment, as described below.

3.7. Cleaning the PFGE equipment

1. Make sure that the pulsed-field electrophoresis chamber is completely drained. For best results, start with the hoses still connected and drain the chamber by attaching a hose to the front of the chamber. Then disconnect

the hose (that connects the chamber to the cooling module) from the front of the chamber and "milk" the hose until it is empty and you see air pushing the remaining liquid away from the cooling module toward the pump (the hose that runs through the cooling module is now empty). Next disconnect the hose from the back of the chamber and drain the remaining hose by alternately lifting the hose and the pump so the liquid drains. Lift the left side of the chamber to drain the subchamber and drain the rest of the liquid through the hose in the front of the chamber by lifting the back end of the chamber.

2. Reattach the hoses to the appropriate ports and add ˜2000 mL of ddH_2O (not autoclaved) into the chamber. Turn on the Control Module and Pump but not the Cooling module, and let the water circulate in the system for 30 minutes.
3. After the chamber is rinsed, drain it (see step 1 for details). Remove all the water from the chamber as well as the hoses. Pat interior dry but avoid touching the electrodes. Leave chamber lid propped open with a wad of paper towels.

SECTION 4 REPORTING and LABELING

All data about isolates, PFGE protocol & steps, amount of reagents used, and gel results (including a picture of the gel) should be kept in your official lab notebook.

SECTION 5 TROUBLESHOOTING

Problems previously encountered with PFGE include:

(1) Smeary patterns usually result from exonuclease contamination in the plug itself or in the digestion reaction. Be careful as not to touch the plug to anything not sterilized or that has not been cleaned with ethanol (i.e. Razor blades, tubes, spatula, etc.)
(2) Smeary patterns can also result from residual fluid sitting in the subchamber. Be sure to completely drain the chamber, subchamber and hoses.
(3) Poor pattern quality; incomplete digest/partials: Use BSA in all digestion reactions; Incubate for longer periods of time (4–6 hrs); not optimal digestion reaction: check digestion calculations and reagents.
(4) Atypical patterns for the references can occur after an electrode has been changed. After changing an electrode, allow silicone sealant to cure for 24 hours, then flush the chamber with circulating dH_2O for several hours.

(5) Weak plugs (that break easily during washes and handling): Use a higher concentration of SKG for making the plugs – protocol for making plugs has been changed (2/13/14) from 0.25g SKG to 0.3g SKG in 23.5mL TE Buffer.

Rationales for modifications to the CDC PulseNet protocol:

(1) Increased OD yields brighter/more intense bands even at low MW.
(2) Increased plug lysis time yields better lysis of plugs, therefore resulting in better quality patterns.
(3) Changes to digestion reaction mixtures: they were based on old CDC PulseNet PFGE protocols and we have found original recipes produce better patterns.
(4) In some instances, enzymes are ordered in high concentration as they are more cost effective. However, number of units per digestion rxn/slice remains constant.
(5) Digest durations have been extended to decrease likelihood of partial/incomplete digests.
(6) Digest durations have been modified a second time (2/13/14) based on Emily's extensive experience.

ADDITIONAL HELP

Below is a link for additional trouble-shooting help made available by PulseNet International:

http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/PFGE_troubleshooting.pdf

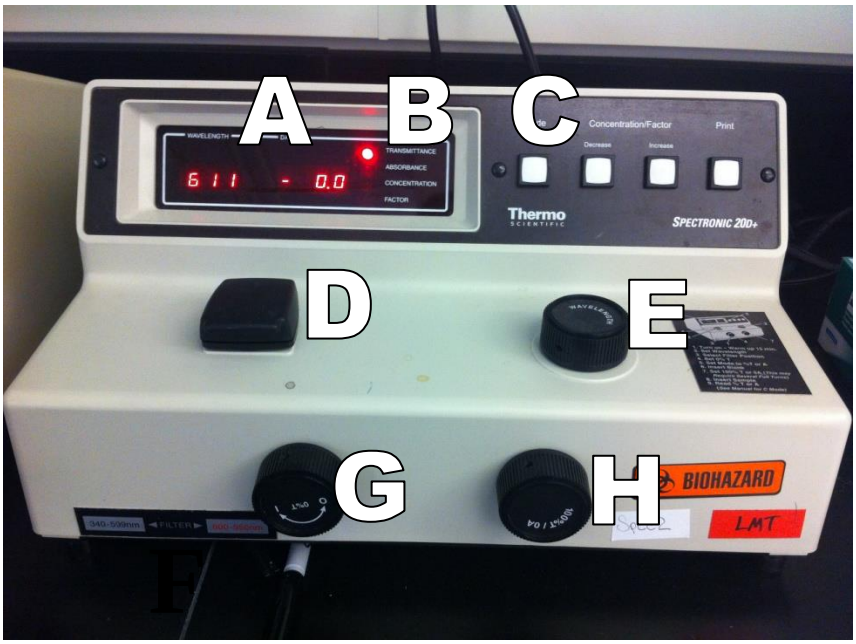
SECTION 6 REFERENCES

1. http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/PNL05_Ec-Sal-ShigPFGEprotocol.pdf
2. **Ribot E.M. et al.** 2006. Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog. Dis*. **3(1)** 59–67.

SECTION 7 APPENDICES

Appendix 1. To measure OD of tubes:

1 Turn on the machine using the left knob (G), let it warm up ~15 minutes.
2 It will start automatically in Transmittance mode (B), the red light is next to the Transmittance option. *Note: It is important to use the appropriate tube holder size in slot D.
3 Change the lever (F) to select the appropriate filter for the wavelength used in your experiment.
4 Use knob (E) to adjust the wavelength on the digital readout (A).
5 With no tube in use (slot D is empty), turn the left knob (G) to set the display (A) to 0.0 (0% Transmittance)
6 Put in your broth/buffer blank tube in slot D and use the right knob (H) to set the Transmittance to 100. (100% Transmittance = 0.000 Absorbance)
7 Push the "Mode" button (C) to change the status from Transmittance to Absorbance. You will see that the "100" on the screen (A) will change to 0.000. The red light moves to



Legend:
A. Digital readout
B. Status indicators
C. Mode
D. Sample compartment (slot)
E. Wavelength control
F. Wavelength filter (lever)
G. Power Switch/Zero control
H. Transmittance/Absorbance control

Appendix 2. PFGE equipment and front panels

Figure 1. PFGE Equipment

CHEF-MAPPER

Electrophoresis Chamber

Control Module

CHEF-DR II

Electrophoresis Chamber

Control Module

Drive Module

Speed pump

Cooling Module

Figure 2. Front panel of Cooling Module

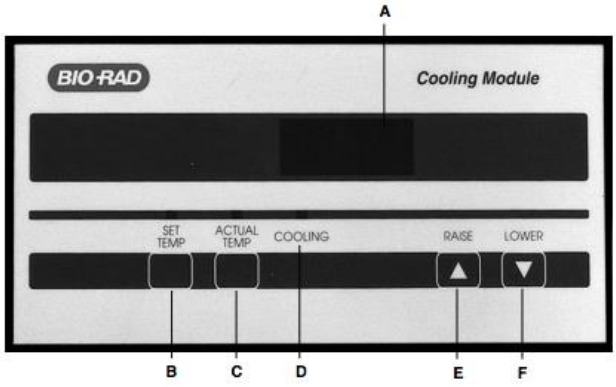
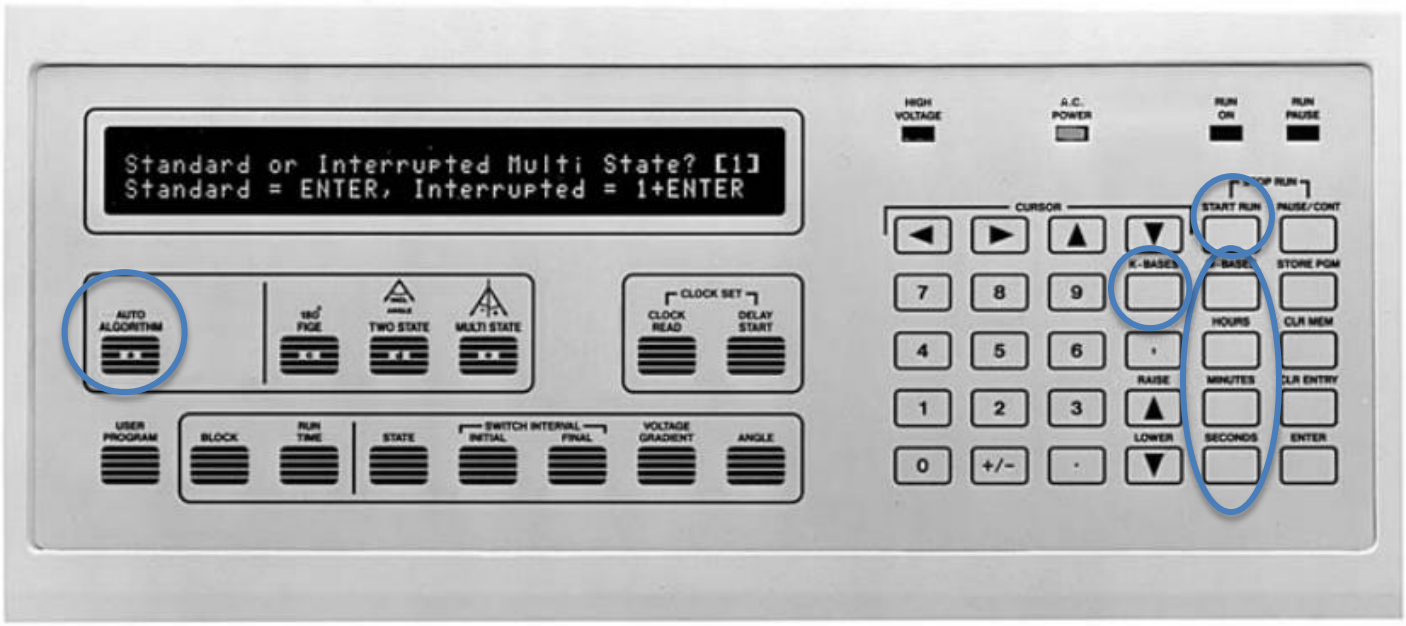


Figure 3. Front panel of the CHEF-DR II

Figure 4. Front panel of the CHEF-Mapper



Appendix 3. Proper disposal of Ethidium Bromide

1. Check the container covered with Aluminum foil to see if it has EtBr solution in it.
2. If it does then make sure one of the two 1000 mL Erlenmeyer flasks with the destaining tea bags inside is empty (it's okay to dump the liquid down the drain if it has been at least 24 hours since EtBr solution was last added) and the tea bag has been used fewer than 7 times, then pour the content of the container into it.
3. After you poured the EtBr into the container, DO NOT forget to write your initials and the date you did it on in the log sheet pasted on the wall.
4. Write your initials and the date you did it.
5. Wait at least 24 hours before dumping the liquid from the flask through the drain.
6. After 7th occasion you used the tea bag (poured the EtBr into the flask and waited at least 24 hours), dispose of the liquid, then take out the tea bag and place it in the container where the rest of used tea bags are.
7. Put a new tea bag inside the flask, and write the date and initials.

Appendix 4. Gel imaging system and software.

Figure 1. GelDoc imaging system from BioRad

Figure 2. Quantity One software icon

Figure 3. First screen within Quantity One software

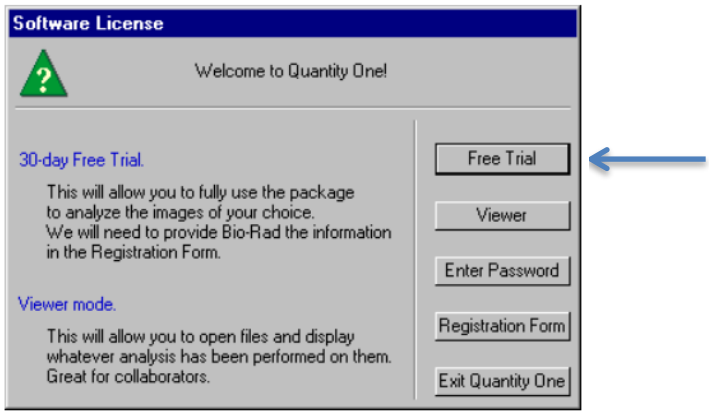
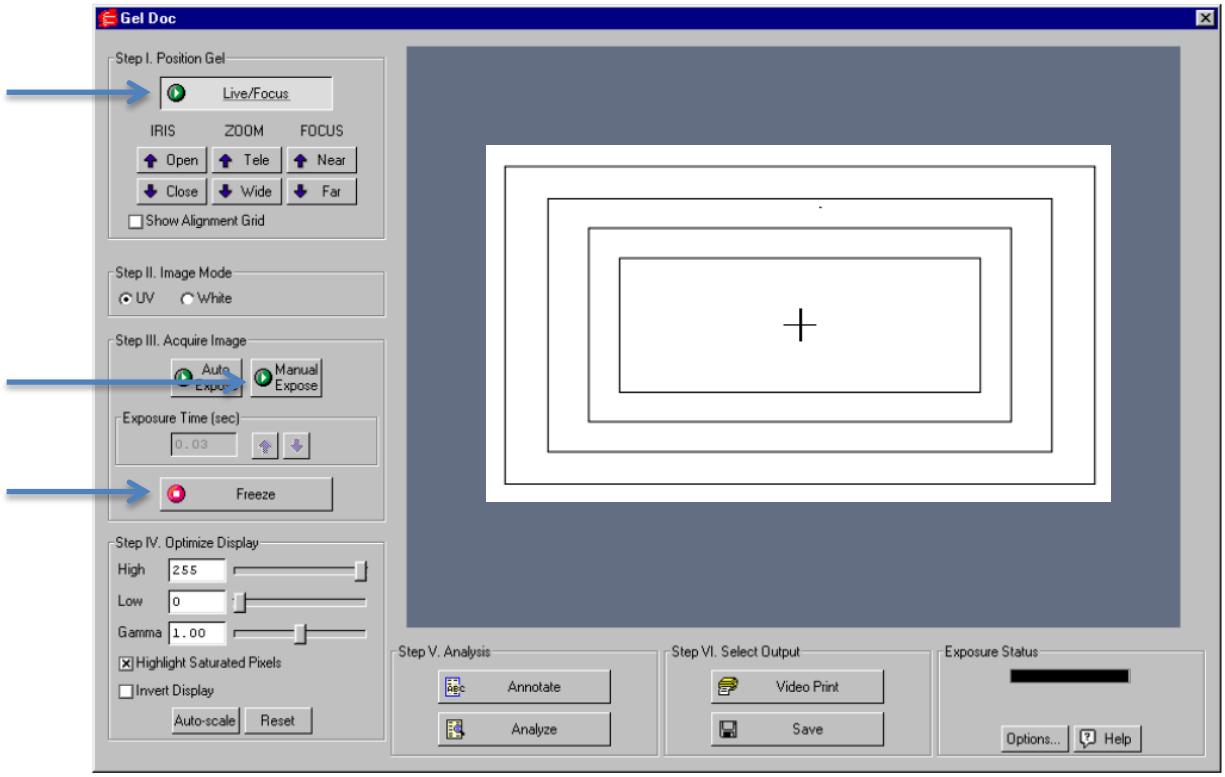


Figure 4. Main Screen within Quantity One software



Appendix 5. Buffer Preparation

Note: All solutions prepared in the Wiedmann lab must be less than 6 months old.

*Freshly-prepared 1M Tris-HCl and 0.5M EDTA do not have to be autoclaved if they are used within 24 hours to prepare TE, CSB, and CLB buffers which will be autoclaved, but they do need to be autoclaved prior to storage.

Bottles to be autoclaved should be less than 2/3 full, therefore, each 1L of buffer should be split between 2 bottles prior to autoclaving.

1. **1M Tris-HCl, pH8.0**
   121.1g Tris Base
   700mL ddH_2O
   80mL 6N HCl
   100mL 6N HCl
   Add 50mL sterile ddH_2O to a sterile 100 mL bottle.
   In the fume hood, slowly add 50 mL 12N HCl (from stock bottle).
   Unused 6N HCl can be added to the stock by the pH meter.
   Allow 6N and 12N HCl to evaporate from all glassware (make sure it's labeled) in the fume hood before rinsing and putting in the wash basin.

Dissolve using hot plate/stirrer at 330 rpm
Adjust pH to 8.00
Adjust volume to 1L
Autoclave*

2. **0.5M EDTA, pH 8.0**
   93.05g EDTA
   12g NaOH
   420mL ddH_2O

Dissolve using hot plate/stirrer at 37^{o}C, 330 rpm
Allow to cool to room temperature
Adjust pH to 8.00
Adjust volume to 500mL
Autoclave*

3. **TE (Tris-EDTA) Buffer (10mM Tris-HCl/1mM EDTA, pH 8.0)**
   10mL 1M Tris-HCl, pH 8.0
   2mL 0.5 M EDTA, pH 8.0
   988mL ddH_2O

Adjust pH to 8.00
Autoclave

4. **Cell Suspension Buffer (CSB) (100mM Tris-HCl/100mM EDTA)**
   75mL 1M Tris-HCl, pH 8.0
   150mL 0.5M EDTA, pH8.0
   525mL ddH_2O
Autoclave

5. **Cell Lysis Buffer (CLB) (50mM Tris-HCl/50mMEDTA/1%Sarcosyl)**
   50mL 1M Tris-HCl, pH 8.0
   100mL 0.5M EDTA, pH 8.0
   10g N-Lauroylsarcosine sodium salt (Sarcosyl)
   750mL ddH_2O
Dissolve using hot plate/stirrer at 50-60^oC, 330 rpm
Adjust volume to 1L
Autoclave

6. **20% SDS (Sodium dodecyl sulfate)**
   20g SDS
   100mL ddH_2O
Dissolve using hot plate/stirrer at 45^oC, 330 rpm

7. **10X TBE (Tris-Borate EDTA) Buffer**
   108g Tris Base
   55g Boric Acid
   9.3g EDTA
   500mL ddH_2O
Dissolve using hot plate/stirrer at 32^oC, 330 rpm
Adjust volume to 1L
Autoclave

8. **0.5X TBE (Tris-Borate EDTA) Buffer**
   5mL 10XTBE
   95mL ddH_2O
Autoclave

9. **1mg/mL EtBr (Ethidium Bromide)**
   5mL 10mg/mL EtBr
   45mL ddH_2O
Wrap bottle with aluminum foil and store in a secondary container inside a drawer to protect from light
Caution: EtBr is a powerful mutagen. Follow EH&S guidelines for disposal of remaining EtBr solution when making a replacement stock after 6 months.

10. **10mg/mL Thiourea**

Move scale into fume hood. Zero using sterile 125ml glass bottle.
Weigh approximately 1g of Thiourea directly into the sterile 125ml glass bottle
Add an appropriate amount of sterile ddH_2O to get a 10mg/mL solution (100mL for 1g)
Wrap bottle with aluminum foil and store in a secondary container inside a drawer to protect from light.
Caution: Thiourea is toxic. Follow EH&S guidelines for disposal of remaining Thiourea solution when making a replacement stock after 6 months.
Note: The amount of Thioruea added to the running buffer was changed from 836□L of stock solution to 860□L of 10mg/mL stock solution on 6/18/14 following review of the current CDC protocol with Martin.

How to pH your buffers

1. Push the button closest to the digital screen to turn on the pH meter, wait until it beeps
2. Push "pH" to put it in pH mode.
3. Rinse probe with ddH_2O, dry with Kimwipe
4. Put probe in 4.0 (pink) pH standard, push "cal", then push downward pointing triangle twice so the pH meter reads "4.01"
5. Push "enter", wait until it beeps, the reading automatically changes to "7.00"
6. Rinse probe with ddH_2O, dry with Kimwipe
7. Put probe in 7.0 (yellow) pH standard, push "enter", wait until it beeps, the reading automatically changes to "10.01"
8. Rinse probe with ddH_2O, dry with Kimwipe
9. Put probe in 10.01 (blue) pH standard, push "enter", wait until it beeps
10. Display will show slope – should be at least 95% (if it is too low, replace the pH standards and recalibrate). Push "Enter" to lock in calibrators.
11. Rinse probe with ddH_2O, dry with Kimwipe
12. Have your buffer stirring at room temperature, put probe in your buffer, push "pH", wait until it beeps
13. Adjust the pH of your buffer up with NaOH or down with HCl
14. Rinse probe with ddH_2O, dry with Kimwipe
15. Have your buffer stirring at room temperature, put probe in your buffer, push "pH", wait until it beeps, repeat steps 11-13 until your buffer reaches the desired pH
16. Rinse probe with ddH_2O, dry with Kimwipe
17. Recheck your buffer's pH: have your buffer stirring at room temperature, put probe in your buffer, push "pH", wait until it beeps, repeat steps 11-15 if necessary
18. Rinse probe with ddH_2O, dry with Kimwipe
19. pH your next buffer, or if you are finished, return the probe to the storage solution and turn off the pH meter by pushing the button closest to the digital screen.