

1. **PURPOSE:** to describe the standardized laboratory protocol for molecular subtyping of Shiga toxin-producing *Escherichia coli* O157 (STEC O157).
2. **SCOPE:** to provide the PulseNet participants with a single protocol for performing MLVA of STEC O157, thus ensuring inter-laboratory comparability of the generated results using the Beckman Coulter CEQ 8000/8800/GeXP platform.

3. DEFINITIONS:

- 3.1. MLVA: Multiple-locus variable-number tandem repeat analysis
- 3.2. VNTR: Variable-number tandem repeat
- 3.3. DNA: Deoxyribonucleic acid
- 3.4. DNase: Deoxyribonuclenase
- 3.5. PCR: Polymerase chain reaction
- 3.6. HPLC: High purity liquid chromatography
- 3.7. dNTP: Deoxyribonucleotide triphosphate
- 3.8. CDC: Centers for Disease Control and Prevention

4. RESPONSIBILITIES/PROCEDURE

4.1. Biosafety warning: Shiga toxin-producing *Escherichia coli* O157 (STEC O157) is a human pathogen causing serious disease. It has been reported that less than 100 cells of STEC O157 may cause infection. Always use a minimum of Biosafety level 2 practices and extreme caution when transferring and handling strains of this serotype. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plastic ware and glassware that come in contact with the cultures in a safe manner.

4.2. Reagents, supplies and equipment needed for DNA template preparation

- 4.2.1. Trypticase soy agar with 5 % sheep blood (TSA-SB) or comparable media
- 4.2.2. 1 µl and 10 µl inoculation loops
- 4.2.3. 0.5 ml microcentrifuge tubes
- 4.2.4. DNase-free, molecular biology -grade water
- 4.2.5. Vortex
- 4.2.6. Boiling water bath or thermal block / thermocycler accommodating 0.5 ml tubes
- 4.2.7. Tabletop centrifuge for high rpm (up to 13,000-14,000 rpm) spinning

4.3. Reagents, supplies and equipment needed for PCR

- 4.3.1. DNA lysates from isolates (store at -20°C or -80°C freezer for long term)
- 4.3.2. PCR primers (see appendix PNL19-1)
 - 4.3.2.1. Fluorescent-labeled forward primers
 - 4.3.2.1.1. HPLC-purified
 - 4.3.2.2. Unlabeled reverse primers
 - 4.3.2.2.1. Regular gel filtration purification
 - 4.3.2.3. Integrated Technologies (Skokie, IL; www.idtdna.com, 1-800-328-2661) and Proligo (Boulder, CO; www.proligo.com, 1-800-234-5362) are currently the suppliers for the Wellred dye-labeled (D2) primers recommended by Beckman Coulter. Biosearch Technologies (Novato, CA; www.biosearchtech.com; 1-800-436-6631) is the

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recommended supplier for the Quasar dye –labeled (Quas670 and 705) primers.

4.3.2.4. Divide the concentrated stocks (100 µM) in portions and store at -80°C freezer

4.3.2.4.1. One vial should contain enough to prepare 25-50 µl of working solution. Avoid repetitive freeze-thaw cycles of concentrated primer stocks.

4.3.2.5. The 1 µM, 2.5 µM, 5 µM and 25 µM working solutions can be stored at either -20°C or -80°C freezer

4.3.2.6. Prepare new working solutions every month or if a significant drop in the fluorescence level is observed (for instructions refer to PNQ05_MLVA CEQ certification, appendix PNQ05-5)

4.3.3. 96-well polypropylene PCR plates (Fisher, Cat. No. 07-200-613) or Microamp PCR tubes without caps (Life Technologies, Cat. No. N8010533)

4.3.4. 8-well strip caps for polypropylene plate (Fisher, Cat. No. 07-200-639) or MicroAmp strip caps for individual tubes (Life Technologies, Cat. No. N8010535)

4.3.5. DNase-free, molecular biology -grade water

4.3.6. 1.5 ml Eppendorf microcentrifuge tubes

4.3.7. PCR Nucleotide mix (ready-to-use dNTP mix containing all four nucleotides; Roche, Cat. No. 11 814 362 001)

4.3.8. Platinum Taq Polymerase with 50 mM MgCl₂ and 10X buffer (Life Technologies Cat. No. 10966-034)

4.3.9. PCR cooling block (VWR International, Cat. No. 62111-762)

4.3.10. DNA Engine (Biorad), GeneAmp (Life Technologies) or similar thermocycler with a heated lid option and 96-well block format

4.3.11. Parafilm M, 4” width (VWR, Cat. No. 52858-032)

4.3.12. Complete set (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl, and 2 µl) of single channel pipettors for mastermix set-up (“clean set”)

4.3.13. A 1-10 µl single channel pipettor for adding DNA templates

4.3.14. Filtered tips for pipettors

4.3.15. Microfuge for low (up to 6,000 rpm) rpm spinning

4.4. Reagents, supplies and equipment needed for Beckman CEQ™ 8000/ 8800/GeXP)

4.4.1. DNase-free, molecular biology -grade water

4.4.2. PCR cooling block (VWR International, Cat. No. 62111-762)

4.4.3. 10 µl, 100 µl, and 1000 µl single channel pipettors

4.4.4. 1-10 µl and 20-200 µl multichannel pipettors

4.4.5. Filtered pipette tips

4.4.6. Sterile solution basins

4.4.7. 1.5 ml Eppendorf microcentrifuge tubes

4.4.8. 5 ml polystyrene round bottom tubes (for example Falcon tube 352058)

4.4.9. 96-well polypropylene (non-PCR) V-bottom plate (for dilutions; Fisher Scientific, Cat. No. 07-200-698)

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- 4.4.10. 96-well Beckman sample plates (Beckman Coulter, Cat. No. 609801; alternative: Fisher Scientific, Cat. No. 07-200-613)
- 4.4.11. 96-well Beckman CEQ buffer plate (Beckman Coulter, Cat. No. 609844; alternative: Fisher Scientific, Cat. No. 07-200-98)
- 4.4.12. Mineral oil (molecular grade) (Sigma, Cat. No. M5904)
- 4.4.13. GenomeLab™ Sample Loading Solution (Beckman Coulter, Cat. No. 608082)
 - 4.4.13.1. After first thawing, divide in portions of 1.0 ml / vial in order to avoid repeated freeze-thaw cycles
- 4.4.14. GenomeLab™ DNA size standard kit 600 bp (Beckman Coulter, Cat. No. 608095)
 - 4.4.14.1. After first thawing, divide in portions of 8 µl / vial in order to avoid repeated freeze-thaw cycles
- 4.4.15. GenomeLab™ Fragment Analysis Test Sample (Beckman Coulter, Cat. No. 608105)
 - 4.4.15.1. Needed only once to establish the system dye color spectra for the instrument
 - 4.4.15.2. Useful also for troubleshooting
- 4.4.16. GenomeLab™ Separation Capillary Array (Beckman Coulter, Cat. No. 608087)
- 4.4.17. GenomeLab™ Separation Buffer, 4x30 ml (Beckman Coulter, Cat. No. 608012)
- 4.4.18. GenomeLab™ Separation Gel LPAI (Beckman Coulter, Cat. No. 608010 for CEQ 8000/single rail GeXP; Cat. No. 391438 for CEQ 8800/double rail GeXP)
- 4.4.19. Parafilm M, 4" width (VWR, Cat. No. 52858-032)
- 4.4.20. A rinse bottle containing distilled water
- 4.4.21. Centrifuge with a microtiter plate rotor
- 4.4.22. Microfuge for low (up to 6,000 rpm) rpm spinning

4.5. Isolate preparation

4.5.1. Day 0

- 4.5.1.1. Streak an isolated colony from pure test cultures to TSA-SB plate (or comparable media). Incubate cultures at 37°C for 14-18 hrs.

4.5.2. Day 1

- 4.5.2.1. For each isolate to be typed, aliquot 100 µl of sterile, molecular biology-grade water into 0.5 ml microfuge tubes. Use a sterile, disposable 1 µl loop to pick 2-3 colonies (about half of a loopfull); rotate the loop in the microfuge tube to release the bacteria into the water. Cap and vortex for 10-15 seconds to disperse any clumps.
- 4.5.2.2. Place the tubes in a 100°C water bath or heat block for 10 minutes. Cool briefly on ice or in fridge and centrifuge for 10 minutes at 10,000 rpm. Place on ice or in fridge while preparing PCR reactions. These DNA templates can be stored at -20°C or -80°C for several years.

4.6. PCR procedure

4.6.1. Day 1

- 4.6.1.1. Fill out, save with the run name, and print a PCR template/CEQ worksheet (see appendix PNL19-2) with appropriately labeled

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samples (a maximum of 43 isolates/plate; 40 unknowns + two positive controls and a negative control; two wells are reserved for the internal ladder; the first column of the plate is reserved for conditioning).

4.6.1.1.1. For each isolate, two wells must be labeled as follows:

BNkeyR1 where “BNkey” represents the isolate-specific state laboratory identification number (be sure to use the exactly same isolate ID that is used in the PFGE gels uploaded to the national database) and “R1” represents one of the two specific multiplex PCR reactions (R1, R2).

4.6.1.1.2. The CEQ™8000/ 8800/GeXP machine has an 8-capillary array and must always be run with a complete column (n=8 wells). For example, 54 wells (6.75 8-well columns) are required to test 26 samples (including controls). The remaining two wells for the seventh column must be filled and run with 20 µl of water or left-over sample loading solution (blanks).

4.6.1.1.3. Also save the CEQ worksheet for the run on a flash drive (memory stick). That way you can easily import the sample plate set-up in the CEQ without having to re-type the sample keys.

4.6.1.2. Fill out, and print a PCR mastermix calculation worksheet (see appendix PNL19-3) by typing the number of isolates to be tested (plus 2-3 extra) in the PCR mastermix calculators labeled R1 and R2. This number is highlighted in red and is next to “number of samples to be analyzed”. The mastermixes for reactions 1 and 2 (R1, R2) for one sample are as follows:

<u>R1</u>	Volume (µl)	Final conc.
PCR water	5.16	
PCR buffer (10x)	1.00	1x
MgCl ₂ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-3F (25 µM)	0.18	0.45 µM
VNTR-3R (25 µM)	0.18	0.45 µM
VNTR-34F (5 µM)	0.28	0.14 µM
VNTR-34R (5 µM)	0.28	0.14 µM
VNTR-9F (5 µM)	0.28	0.14 µM
VNTR-9R (5 µM)	0.28	0.14 µM
VNTR-25F (2.5 µM)	0.28	0.07µM
VNTR-25R (2.5 µM)	0.28	0.07 µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

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<u>R2</u>	Volume (µl)	Final conc.
PCR water	5.96	
PCR buffer (10x)	1.00	1x
MgCl ₂ (50 mM)	0.40	2.00 mM
dNTPs (10 mM)	0.20	0.20 mM
VNTR-17F (5 µM)	0.18	0.09 µM
VNTR-17R (5 µM)	0.18	0.09 µM
VNTR-19F (1 µM)	0.20	0.02µM
VNTR-19R (1 µM)	0.20	0.02 µM
VNTR-36F (1 µM)	0.12	0.012 µM
VNTR-36R (1 µM)	0.12	0.012 µM
VNTR-37F (2.5 µM)	0.12	0.03µM
VNTR-37R (2.5 µM)	0.12	0.03µM
Taq (5 U/µl)	0.20	1 U
	= 9.00	

4.6.1.2.1. **NOTE: NOTE:** these primer concentrations serve as a starting point. Since laboratory-specific factors, such as the age of the primer stocks, calibration status of the thermocyclers and pipettes, etc. affect amplification efficiency, each laboratory will have to re-optimize the primer concentrations for optimal detection of all targets. However, any other parameters stated in the SOP should not be changed.

4.6.1.3. Thaw all reagents and supplies needed for PCR reactions and place on ice; keep primers light protected as much as possible.

4.6.1.3.1. PCR mastermixes should be set up in a clean hood that is dedicated just for this purpose and where no cultures or DNA are handled.

4.6.1.4. Prepare the two separate PCR mastermixes in 1.5 ml Eppendorf tubes following the instructions in the PCR mastermix calculation worksheet (see appendix PNL19-3) and place on ice. Add the mastermix components in the following order: water, 10x PCR buffer, Mg₂Cl, dNTPs, primers, and then finally Taq polymerase. Mix the reaction mixture by vortexing briefly.

4.6.1.4.1. Vortex all reagents except Taq polymerase before adding to the mastermix. Taq may be briefly centrifuged with low rpm, if necessary, to pull the enzyme down to the bottom of the tube.

4.6.1.5. Place a 96-well PCR plate or required number of PCR tubes in a PCR cooling block.

4.6.1.6. Dispense 9.0 µl of each mastermix into the appropriate columns of the 96-well polypropylene plate / PCR tubes as noted in the PCR template/CEQ worksheet (see appendix PNL19-2)

4.6.1.7. Add 1 µl of PCR water to each of the two different wells representing the negative controls of the two reactions.

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4.6.1.8. Add 1.0 µl of DNA lysate to each of the two different wells representing the two PCR reactions. Use STEC O157 strain EDL933 (ATCC 43895) as a positive control (it is recommended to run the positive control in duplicate). The internal ladder to be used will be comprised of pooled PCR products of the isolates EC04PN0139 and EC04PN0570 (see appendix PNL19-5 for instructions for ladder preparation)

4.6.1.9. Cover all wells / tubes with 8-well strip caps and firmly clamp down to avoid any evaporation during PCR amplification.

4.6.1.10. Recommendation: briefly spin down the plate / tubes to remove any air bubbles.

4.6.1.11. Setup the following PCR parameters and save the program as “O157MLVA”:

- * 95°C for 5 min Step 1
- * 94°C for 20 sec Step 2
- * 65°C for 20 sec Step 3
- * 72°C for 20 sec Step 4
- * Go to step 2, 34x Step 5
- * 72°C for 5 min Step 6
- * Indefinite hold at 4°C

4.6.1.11.1. Make sure to use the heated lid option on the PCR block and “calculated” or tube control (instead of block or probe control) as a temperature control method.

4.6.1.12. When the PCR is complete, store the amplification products light-protected at 4°C until ready to run on CEQ/GeXP. If the fragment analysis is not performed the same day, the plate should be stored at -20°C or -80°C. The PCR products are stable for approximately one month, when stored frozen.

4.7. Initial setup of CEQ™8000/ 8800/GeXP instrument

4.7.1. **NOTE:** Steps 4.7.2.-4.7.8. only need to be performed before the very first run and every time a new working database is created.

4.7.2. In the main page of the CEQ software version 8.0 or 9.0 or the GeXP software version 10.2, click on the “Fragments” icon. Select “Analysis” in the bar at the top of the window to open a window called “Analysis Parameters”.

4.7.3. Select “DefaultFragmentAnalysisParameters” in the top drop-down menu and click “Edit”. In the appearing “Edit Fragment Analysis Parameters” window, select the tab “Analysis Method” and select “Size Standard 600” for size standard and “Quartic” for model. Do not make any other changes.

4.7.4. Click on “Save As” and name the analysis parameter as “MLVA600”.

4.7.5. Go back to the main page of the CEQ program and click on the “Set Up” icon. Select “Create a New Sample Plate” and click “OK”.

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- 4.7.6. In the “Sample Setup” window, check to be sure that the methods “Condition” and “Frag-test” are present in the drop-down menu below each column. These should be the instrument default methods.
- 4.7.7. If they are not present or to create the frag-test_pause method, highlight any of the default methods and select “Method” tab from below. This will open a “Method” window. Click on the “Edit” button in this open window and put in the following parameters in the “CEQ Methods” table below.

CEQ Methods:

		Condition	Frag-test_pause
Capillary	Temperature	35	35
	Wait	0	Yes
Denature	Temperature	90	90
	Time	0	120 seconds
Pause	Time	NA	10 min
Inject	Voltage	2.0 kV	2.0 kV
	Time	30 seconds	15 seconds
Separation	Voltage	7.5 kV	6.0 kV
	Time	35 minutes	60 minutes
Pause		0 minutes	0 minutes

- 4.7.8. Be sure to save the new method with new file names. Each time a new CEQ database is generated, these methods are not copied. However, if these methods were in the machine as a default (Frag-test_pause is not), they are copied when a new database is created.

4.8. CEQ™8000/ 8800/GeXP instrument preparation before each run

4.8.1. Day 1

4.8.1.1. Make sure a capillary array is installed in the instrument. For installation, follow the instructions in the appendix PNL19-4.

4.8.1.1.1. If the instrument is used frequently, there is no need to remove the array after every run. Beckman Coulter guarantees the shelf life of the array for up to 30 days (or up to 100 runs) at room temperature. However, as long as the positive control and the internal ladder fall within the sizing range indicated in the appendices PNL19-2 and PNL19-5 and the fragment analysis failure rate is less than 5 %, the same capillary array can be used up to 45 to 50 days.

4.8.1.2. To setup the plate run on the CEQ\GeXP, click on the “Set Up” icon on the CEQ\GeXP main page and select “Create a New Sample Plate” and click “OK”.

4.8.1.3. Using a USB flash drive, open the PCR/CEQ Excel worksheet (PNL19-2) saved for this plate run and copy only the 96-well format and paste it directly into the blank sample setup window.

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- 4.8.1.4. Under the first column of the plate format, select the method “Condition” from the drop-down menu. Select the method “Frag-test_pause” for the columns containing the samples.
- 4.8.1.5. Highlight all columns containing samples. At the bottom of the screen, select the “Analysis” tab, check the box for “Perform analysis”, and select the “MLVA600” parameter set from the drop-down menu.
- 4.8.1.6. **NOTE:** The export tab does not contain the correct format (CSV) and should be left unchecked.
- 4.8.1.7. Save the plate with a run name following the standardized PulseNet naming system: Use the unique identifier code that was assigned to your laboratory by PulseNet for the first two to four letters of the file name. The next two spaces indicate the year and the next four spaces indicate the month and the date the run was performed. For example GA070426 is a run made at the GA Public Health Laboratory on April 26th 2007. If several runs are performed the same day, separate the file names by using sequential numbers, for example GA070426-1, GA070426-2.
- 4.8.1.8. Close “Set Up” module.
- 4.8.1.9. Click on the “Run” icon in the main CEQ\GeXP window. The “Run Control – CEQ System” window will appear.
- 4.8.1.10. If the instrument does not have a gel cartridge installed, the “Instrument Warning Message” window will appear stating “Instrument Status: Loading Gel Cartridge”. Click “OK”.
- 4.8.1.11. If the instrument already has a gel cartridge installed, the above message does not appear. In this case, you can check the amount of gel available by clicking on the “Life” tab on the left side of the “Run Control – CEQ System” window. If a new gel cartridge needs to be installed, select “Release Gel Cartridge” from the “Replenish” drop-down menu or from the toolbar and wait for the CEQ\GeXP to display a green “Go” signal box.
 - 4.8.1.11.1. **NOTE:** A full plate uses approximately 9.0 ml of gel.
- 4.8.1.12. Open the gel access door, pull out the yellow plug or the old gel cartridge and install the new gel cartridge. Select “Install Gel Cartridge” from the “Replenish” drop-down menu or from the tool bar. The “Install Gel Cartridge” dialog box will open. Type in the lot number for the gel, click on “Set To New” to zero out the time on the instrument (if the cartridge is new) or type in the number of hours the gel cartridge (previously used) has spent in the instrument. For the GeXP, designate whether the gel cartridge is ‘New’ or ‘Used’ by selecting the corresponding radial button. If installing a previously used cartridge, enter the number of hours on instrument. When finished click on “Done”.
- 4.8.1.13. Perform a manual purge by selecting “Manifold Purge” from the “Direct Control” drop-down menu or by clicking on the funnel on the

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direct control screen. A “Manifold Purge” dialog box will open. Set 0.1 ml as the purge volume and 2 as the number of purge cycles and click on “Purge”.

4.8.1.13.1. **NOTE:** a manifold purge is only necessary when a new/used gel cartridge is inserted. If using a cartridge already installed in the instrument, a manifold purge is not necessary.

4.9. Fragment analysis sample preparation

4.9.1. Day 1

4.9.1.1. Thaw the CEQ sample loading solution (SLS), 600 bp DNA size standard and internal ladder (see appendix PNL19-5 for preparation instructions) and place on ice.

4.9.1.2. While the reagents are thawing, prepare a Beckman buffer plate by filling a required amount of wells about ¾ full (~250 µl) with CEQ Separation Buffer.

4.9.1.3. Prepare a 96-well V-bottom plate for diluting the PCR reactions. Using a 200 µl multichannel pipettor and a solution basin, dispense 59 µl of molecular-grade water in the required number of wells.

4.9.1.4. Remove the plate / tubes with the PCR reactions from the thermocycler. Briefly spin down the plate / tubes, if necessary. Use a 10 µl multichannel pipettor to transfer 1 µl of each PCR reaction directly across to the corresponding set of wells in the dilution plate. In order to avoid cross-contamination, remove the strip cap from just one column at a time and recap the column before opening the next one.

4.9.1.5. For the internal ladder, combine R1 and R2 PCR products from the four PCR reactions of both internal ladder isolates into one tube to end with a total of 40 µl. Mix well by pipetting up and down a few times and add 3 µl of internal ladder in two wells.

4.9.1.6. Using a 200 µl multichannel pipettor, mix the dilutions by pipetting up and down a few times. Cover the plate with Parafilm and put in the fridge or on ice.

4.9.1.7. Prepare a fragment analysis master mix containing DNA size standard and SLS buffer for the samples following the calculations indicated in the table below. The fragment analysis mastermix calculations can also be performed using the autocalculate box at the bottom of the PCR/CEQ worksheet (see appendix PNL19-2). Vortex briefly and place on ice.

Reagents	Frag. anal. mastermix
CEQ SLS buffer	20 µl x (# samples +3)=
CEQ 600 bp size standard	0.08 µl x (# samples +3)=

4.9.1.8. Place a Beckman 96-well sample plate in a cold block. Add two drops of separation buffer to each well of the column 1 (conditioning

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lane). Next, aliquot 20 µl of the prepared fragment analysis mastermix to the required number of wells. Cover the plate loosely with Parafilm.

- 4.9.1.9. Using the 10 µl multichannel pipettor, add 1 µl of 1:60 diluted PCR reactions to the appropriate columns in the Beckman sample plate. Keep sliding the Parafilm sheet from column to column to keep track of the sample order.
- 4.9.1.10. Briefly (15 s) spin down the CEQ Beckman sample plate to remove any air bubbles.
- 4.9.1.11. Overlay the wells containing samples with 1 drop of mineral oil.
- 4.9.1.12. Select “Start Sample Plate” from the “Run” pull-down menu or click on the “Run” button in the toolbar.
 - 4.9.1.12.1. For the CEQ 8000, a “Select the Sample Plate to Run” window will open. Select the plate layout that was saved above. The “Confirm Configuration-(*Sample Plate Name*)” window will pop up. Confirm that the plate layout matches with the lanes highlighted in the plate configuration window and click on “Load Plates”. When the “Access Plates” dialog box appears, click on “Start”. Wait for the CEQ to display the green “Go” signal box.
 - 4.9.1.12.2. For the GeXP, a “Sample Plate Run Confirmation” window will open. This offers the option to designate operator’s name. At the bottom of the window, select “Sample Plate Name” and select the plate layout that was previously saved above. Confirm that the plate layout matches with the lanes highlighted in the plate configuration window and that the plate location is correct. If running two plates, designate which plate to run first by selecting the corresponding radial button. (This window also offers the option to replenish the gel cartridge at this time by selecting the “Install Gel Cartridge” button at the bottom of the window and follow instructions as previously described in 4.8.1.12). Click on “Load Plates”. When the “Access Plates” dialog box appears, click on “Start”. Wait for the GeXP to display the green “Go” signal box.
- 4.9.1.13. Lift the sample access cover. Rinse and refill wetting tray(s) with dH₂O. (If setting up on the GeXP the corresponding wetting tray for each rail must be filled if running two plates.) Dry off outer tray, clean off any dried deposits of gel from the lid and place the wetting tray(s) back on machine.
- 4.9.1.14. Load the buffer plate(s) on the front tray and the sample plate(s) on the back tray with notched corners top right. Close the sample access cover. Click on “Load”. This will take you back to the “Sample Plate Run Confirmation” window.
- 4.9.1.15. Once the instrument has loaded the plates, the “Start” button will be highlighted and can be clicked to start the run.

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4.9.1.16. Running one column (8 wells) takes about 105 min. A full plate run takes 19½ h. At end of the run, the data will be automatically analyzed and saved.

4.10. Data export from the Beckman Coulter CEQ™ 8000/ 8800/GeXP

4.10.1. Day 2

4.10.1.1. To view the analyzed data, click on the “Fragments” icon in the main CEQ window and a window labeled “Study” will open up.

4.10.1.1.1. If the “Study” window does not pop up, select “New Study” from the “File” drop-down menu.

4.10.1.2. Select the “Analyzed Results” button and then select the desired plate run listed in the window and click “OK”. The “Select Result Data” window will open.

4.10.1.3. In the “Select Result Data” window, select the “Plate View” tab and the plate format will pop up. Select the desired wells for viewing from the plate by either clicking on them individually or select a whole column by clicking on the column number. Selected wells will be highlighted. Complete the sample selection by clicking on the “Finish” button. “Fragment Analysis – New Study” window will open with all the selected wells listed under the “Results” section.

4.10.1.4. Check the fragment result data (the fluorescent peaks) for each well by clicking on the well IDs individually.

4.10.1.4.1. Make sure that all eight VNTRs amplified in the EDL933 control and that the fragment sizes are within the range specified in the appendix PNL19-2 and record the fragment sizes on the PCR/CEQ worksheet.

4.10.1.4.2. The size calling for the internal ladder should also be within the range specified in the appendix PNL19-2 (or PNL19-5).

4.10.1.4.3. Write down any failed reactions in the PCR/CEQ worksheet. Make a note of non-specific peaks, primer-dimers, and atypical appearance of the DNA size standard peaks.

4.10.1.4.4. If the molecular size standard does not run all the way till the end (640 bp) or if it skips fragments, a reaction is considered “a fragment analysis failure” and should be re-run.

4.10.1.4.5. Low fluorescence level background peaks can sometimes interfere with data analysis in BioNumerics. They can be filtered out by using the following procedure:

4.10.1.4.5.1 From the “Study explorer” field on the top left corner, click on “Fragment list”. “Exclusion filter set” is located in the right top corner.

4.10.1.4.5.2 From the “Name” drop-down menu, select the “pk height (rfu)” option.

4.10.1.4.5.3 From the “Operators” drop-down menu select the “<” option. Type in “1000” (or the fluorescence unit value you have determined is adequate to filter out the background

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without eliminating the true positive peaks) in the “Value(s)” field.

4.10.1.4.5.4 Click on “Apply”.

4.10.1.5. Save the study: from “File” drop-down menu, select the option “Save Study As”. “Save Study As” dialog box will appear. Name the study with the standardized run name (for example, “GA070426”) and click “OK”. This ensures that you don’t have to re-analyze the samples if you want to go to check the raw data again later.

4.10.1.6. From the “File” drop-down menu, select the option “Export Fragments/Genotypes”. “Export Fragments/Genotypes” dialog box will appear. Choose the location you would like the file to be saved to (examples: E:/Compact Disk or F:/Removable Disk). Name the peak file containing the fragment list with the run name (see section 4.8.1.7. for instructions; for example “GA070426”), make sure that the file type is “CSV (comma limited)” and save it by clicking on “Save” button. “Export Status” dialog box will appear. Once the export is completed, “OK” button will be highlighted and can be clicked.

4.10.1.7. The remaining gel can stay in the instrument if it is going to be used within 48 hours. The shelf-life of the gel at the room temperature is 72 hours. Using an expired gel may cause inaccurate sizing of fragments in some loci.

5. FLOW CHART:

6. BIBLIOGRAPHY:

- 6.1. Hyytiä-Trees, E., Smole, S. C., Fields, P. I., Swaminathan, B., and Ribot, E. M. (2006) Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis (MLVA) of Shiga toxin-producing *Escherichia coli* O157 (STEC O157). Foodborne Pathog. Dis. 3, 118-131.
- 6.2. Hyytiä-Trees, E., Lafon, P., Vauterin, P., and Ribot, E. (2010) Multi-laboratory validation study of standardized multiple-locus VNTR analysis (MLVA) protocol for Shiga toxin-producing *Escherichia coli* O157 (STEC O157): a novel approach to normalize fragment size data between capillary electrophoresis platforms. Foodborne Path. Dis. 7, 129-136.

7. CONTACTS:

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8. AMENDMENTS:

1/27/2009: Separation buffer is to be used for conditioning instead of distilled water (step 4.9.1.8)

10/6/2009: statement was added to the step 4.10.1.7. that using expired gel may cause inaccurate fragment sizing.

6/8/2011: Instructions for operating the new GeXP version of the Beckman Coulter Genetic Analysis system were added.

4/10/2012: “Frag-test” CEQ running conditions were replaced with “Frag-test_pause” conditions

4/4/2013: former appendix PNL19-4 (BioNumerics specifications for the *E. coli* O157 VNTR loci) was moved to SOP PND14 (PulseNet standard operating procedure for analysis of MLVA data of Shiga toxin-producing *Escherichia coli* in BioNumerics – Beckman Coulter CEQ 8000/8800/GeXP data). Former appendices PNL19-5 and PNL19-6 were renamed PNL19-4 and PNL19-5, respectively.

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Appendix PNL19-1

STEC O157 MLVA PCR Primers

Locus ¹	Dye ²	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
R1			
VNTR-3	D2	GG CGG TAA GGA CAA CGG GGT GTT TGA ATT G	GAA CAA CCT AAA ACC CGC CTC GCC ATC G
VNTR-34	Quas670	GA CAA GGT TCT GGC GTG TTA CCA ACG G	GTT ACA ACT CAC CTG CGA ATT TTT TAA GTC CC
VNTR-9	Quas670	GC GCT GGT TTA GCC ATC GCC TTC TTC C	GTG TCA GGT GAG CTA CAG CCC GCT TAC GCT C
VNTR-25	Quas705	GC CGG AGG AGG GTG ATG AGC GGT TAT ATT TAG TG	GCG CTG AAA AGA CAT TCT CTG TTT GGT TTA CAC GAC
R2			
VNTR-17	D2	GC AGT TGC TCG GTT TTA ACA TTG CAG TGA TGA	GGA AAT GGT TTA CAT GAG TTT GAC GAT GGC GAT C
VNTR-19	Quas670	GC AGT GAT CAT TAT TAG CAC CGC TTT CTG GAT GTT C	GGG GCA GGG AAT AAG GCC ACC TGT TAA GC
VNTR-36	Quas670	GG CGT CCT TCA TCG GCC TGT CCG TTA AAC	GCC GCT GAA AGC CCA CAC CAT GC
VNTR-37	Quas705	GC CGC CCC TTA CAT TAC GCG GAC ATT C	GCA GGA GAA CAA CAA AAC AGA CAG TAA TCA GAG CAG C

¹Primer sets are listed as multiplex PCR cocktails.

²Wellred dye used for D2 (black); Quas705 and Quas670 used as more inexpensive replacements for Wellred D3 (green); and D4 (blue). Only the 5' end of the forward primer is labeled.

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**LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET
MLVA OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O157 (STEC
O157) – BECKMAN COULTER CEQ 8000/8800/GeXP PLATFORM**

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Appendix PNL19-2

STEC O157 MLVA PCR template and CEQ Worksheet

PCR run date: _____
 PCR instrument: _____
 CEQ run date: _____
 CEQ instrument: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	blank											
C	blank											
D	blank											
E	blank											
F	blank											
G	blank											
H	blank											

	1	[put in # of samples only]
SLS	80	
600 bp	0.32	

SLS lot no.
 600 bp lot no.
 Gel lot no.
 Buffer lot no.

<i>E. coli</i> O157 - Positive Control (EDL933)			
R1 fragment sizes		R2 fragment sizes	
VNTR 25 (134-136)		VNTR 17 (156-159)	
VNTR 34 (278-280)		VNTR 36 (158-161)	
VNTR 3 (374-377)		VNTR 37 (187-191)	
VNTR 9 (530-533)		VNTR 19 (307-311)	

- For each isolate, fill in an appropriate BioNumerics key number for PCR reactions R1 and R2.
 (example: for an isolate CDC_K1720 the BN key numbers are CDC_K1720R1 and CDC_K1720R2).
- Add 2 drops of separation buffer in each well in column 1.
- Fill in the exact number of fragment analysis reactions (2 per isolate) in the red box (extra already included in calculation).
 Mix the Sample Loading Solution (SLS) and 600 bp ladder in an appropriate tube. Add 20 µl of mixture to each well.
- Using a multichannel pipettor, add 1 µl of 1:60 diluted PCR reaction to wells containing 20 µl of SLS/600bp ladder mixture.
- Briefly spin down the plate.
- Overlay all wells, except the blank, with 1 drop mineral oil. Immediately place on CEQ or store at -80C.

<i>E. coli</i> O157 - Internal Ladder fragment sizes			
	Expected Size Ranges	Fragment Sizes	
VNTR 25	122-124	134-136	
VNTR 17	149-152	175-178	
VNTR 36	172-175		
VNTR 37	194-197	200-203	
VNTR 34	224-227	260-262	
VNTR 19	296-299	320-322	
VNTR 3	392-395	429-431	
VNTR 9	518-521	566-570	

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**LABORATORY STANDARD OPERATING PROCEDURE FOR PULSENET
MLVA OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O157 (STEC
O157) – BECKMAN COULTER CEQ 8000/8800/GeXP PLATFORM**

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Note: This is posted on the WebBoard as an Excel file so users may save it locally, enter data and use the auto calculate function

PCR Mastermix calculations

Type in the number of reactions (+ 2 extra), currently a red one, next to "Number to analyze" for autocalculations.

Date:

Technician PCL

Appendix PNL19-3

E. coli multiplex PCR		R1			
1	Number of samples to be analyzed				
10	ul Total Reaction Volume				
		Start	Final	Add	
	PCR Buffer	10 X	1 X	1.00 ul	
	MgCl2	50 mM	2 mM	0.40 ul	
	dNTPs	10 mM	0.2 mM	0.20 ul	
black	WNTR-3F	25 uM	0.45 uM	0.18 ul	
	WNTR-3R	25 uM	0.45 uM	0.18 ul	
blue	WNTR-34F	5 uM	0.14 uM	0.28 ul	
	WNTR-34R	5 uM	0.14 uM	0.28 ul	
blue	WNTR-6F	5 uM	0.14 uM	0.28 ul	
	WNTR-6R	5 uM	0.14 uM	0.28 ul	
green	WNTR-25F	2.5 uM	0.07 uM	0.38 ul	
	WNTR-25R	2.5 uM	0.07 uM	0.38 ul	
	Platinum Taq	5 U/ul	1 U	0.20 ul	
	PCR Water			5.16 ul	

	Lane 1	Lane 2	Lane 3
A			
B			
C			
D			
E			
F			
G			
H			

	Date	PCR Reagents	Lot#	Exp.
WNTR-3F		TOX Buffer		
WNTR-3R		MgCl2		
WNTR-34F		dNTPs		
WNTR-34R		Platinum Taq		
WNTR-6F				
WNTR-6R				
WNTR-25F				
WNTR-25R				

E. coli multiplex PCR		R2			
1	Number of samples to be analyzed				
10	ul Total Reaction Volume				
		Start	Final	Add	
	PCR Buffer	10 X	1 X	1.00 ul	
	MgCl2	50 mM	2 mM	0.40 ul	
	dNTPs	10 mM	0.2 mM	0.20 ul	
black	WNTR-17F	5 uM	0.09 uM	0.18 ul	
	WNTR-17R	5 uM	0.09 uM	0.18 ul	
blue	WNTR-19F	1 uM	0.02 uM	0.20 ul	
	WNTR-19R	1 uM	0.02 uM	0.20 ul	
blue	WNTR-36F	1 uM	0.012 uM	0.12 ul	
	WNTR-36R	1 uM	0.012 uM	0.12 ul	
green	WNTR-37F	2.5 uM	0.03 uM	0.12 ul	
	WNTR-37R	2.5 uM	0.03 uM	0.12 ul	
	Platinum Taq	5 U/ul	1 U	0.20 ul	
	PCR Water			5.98 ul	

	Lane 3	Lane 4	Lane 5
A			
B			
C			
D			
E			
F			
G			
H			

WNTR-17F	
WNTR-17R	
WNTR-19F	
WNTR-19R	
WNTR-36F	
WNTR-36R	
WNTR-37F	
WNTR-37R	

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Appendix PNL19-4

Maintenance of the Beckman Coulter CEQ™ 8000/ 8800/GeXP

After each run

1. Clean the wetting tray thoroughly by first rinsing it with warm tap water and then with distilled water. Remove any dried gel from the tray and the lid with a paper towel. Refill the tray with distilled water.
2. The remaining gel can stay in the instrument if it is going to be used within 48 hours. The shelf-life of the gel at the room temperature is 72 hours.

Instrument is not used for a week or more

1. Refill the wetting tray at least twice a week with distilled water to keep the capillaries moist at all times.
2. It is recommended to keep a gel in the injection pump at all times to keep the pump moist. Use leftover gels (< 3.0 ml) that cannot be used for a run anymore.
3. Make a manual purge (0.1 ml 4 times) once a week to keep the lines open and the capillaries unblocked.
4. Remove the capillary array if the instrument is not used for more than two weeks.

Instructions to change the Beckman CEQ capillary array

1. Open the new capillary array package and carefully work loose the plastic covers from the electrode block, but leave the covers still attached.
2. To release the old capillary array, select the “Release Capillary Array” from the “Run” pull down menu or click on the capillary array picture in the direct control screen → the “Remove Capillary Array” dialog box will appear
3. Open the sample access cover
4. Open the capillary access cover
5. To open the capillary temperature control cover, unlatch the two rubber latches
6. To open the manifold access cover and the plenum assembly, loosen the captive screws
7. Lift the red “eject lever” to release the array fitting
8. Pull out the array fitting with your right hand and the electrode block with your left hand and set aside
9. Remove the plastic covers from the electrode block and the array fitting of the new array. Be careful not to break the capillaries!
10. Remove the yellow lens cover from the array fitting. Be careful not to touch the lens!
11. Align the array fitting and the electrode block with the guide pins.
12. Replace the manifold access cover and the plenum assembly. Carefully route the capillaries through the whole in the plenum assembly.
13. Close the capillary temperature control cover.
14. Lower the capillary access cover and the sample access cover to their locking positions

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15. Click on “OK” in the “Remove Capillary Array” dialog box → the “Install Capillary Array” dialog box will appear
16. Change the serial number and click first on “Set to New” and then on “Done” → the “Confirm Capillary Array Selection” dialog box will appear → select “Yes”
17. Make sure that the instrument has a gel cartridge and a wetting tray in place. Select “Capillary Fill” from the “Direct Control” drop-down menu. Let the instrument fill the capillaries (take’s about 3.7 min).
18. Repeat the capillary fill step.
19. Perform three manual purge cycles with 0.1 to 0.4 ml of gel by selecting “Manifold Purge” from the “Direct Control” drop-down menu or by clicking on the funnel on the direct control screen.
20. To perform the optical alignment, either select “Optical Alignment” from the “Direct Control” drop-down menu or click on the lens picture in the direct control screen
21. After the optical alignment has been completed, monitor the fluorescence baseline: select “Monitor Baseline” from the “Run” drop-down menu → “Monitor Baseline” dialog box will appear → check the “Enable Monitor Baseline” box and click “OK”
22. To view the baseline trace, switch to data monitor window and view the baseline for each capillary. Baseline should be less than 5000 units. If it is higher, perform the following procedure:
 - a. Release the capillary array and open the sample access cover, the capillary access cover, the temperature control cover and the manifold access cover
 - b. Remove the array fitting
 - c. Carefully wipe clean both sides of the optical window in the array fitting by using a cotton swab moistened with distilled water. Wipe to one direction only.
 - d. Dry the optical window using a second swab by wiping to the same direction as above
 - e. Clean the manifold access area first by using a moist swab and then with a dry swab
 - f. Replace the array fitting in the manifold
 - g. Click on “OK” in the “Remove Capillary Array” dialog box → the “Install Capillary Array” dialog box will appear
 - h. Select “Clean Capillaries” button, and click on “Done”
 - i. Perform two capillary fills and three manual purges
 - j. Repeat the optical alignment step. If the baseline is still above 5000 units after the repeated optical alignment, the new capillary array is most likely defective and must be replaced with another array.
23. To stop the baseline monitoring, go back to the “Monitor Baseline” dialog box and uncheck the “Enable Monitor Baseline” box and click on “OK”
24. To check the optical scan data, go the Main menu and click on the “Sequencing” icon. From the “File” drop-down menu, select “Open” → the “Open” dialog box will appear → select the “Optical scan data” tab. Find today’s optical scan file by using the filter (type in today’s date as a start and an end date and click on “Refresh”) → highlight today’s scan file and click on “OK”. The red and blue peaks in the optical scan data should start from the galvanometer position 45-50 and end at about 200-210. If the peaks start much earlier or later, the optical alignment of the instrument is off and may contribute to increased fragment analysis failure rate. The red and blue peaks should be superimposed at each galvanometer reading. If they are clearly separated, the optical alignment is not optimal. In either case, a service call to the Beckman technical support is needed.

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Instructions to make a new working database

1. Close off all open modules.
2. In the main menu of the CEQ software, click on the “Database” icon.
3. From the “File” drop-down menu, select “New Database” → The “New Database” dialog box will appear
4. Enter a name for the new database by indicating the date on which it was created. For example: Oct_6_2006.
5. Check the box “Set as a Working Database” and click “OK”.

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Appendix PNL19-5

Instructions to prepare the internal ladder

1. Prepare DNA templates from isolates EC04PN0139 and EC04PN0570 as described in the protocol step 4.5. Store the templates at -20°C or -80°C freezer.
2. Use the DNA templates to set up and run the PCR reactions R1 and R2 as described in the protocol step 4.6.
3. After the PCR amplification, pool the R1 and R2 reactions for isolates EC04PN0139 and EC04PN0570 into one single PCR tube to gain a total volume of 40 µl.
4. Each new lot of internal ladder must be tested against the old ladder lot by running them in the same fragment analysis run.
5. Store the ladder in -20°C or -80°C freezer. It should remain stable at least 5-6 freeze-thaw cycles for a period of one month.

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the internal ladder as seen in the electropherogram from left to right:

VNTR-25 (D3):	122-124	134-136
VNTR-17 (D2):	149-152	175-178
VNTR-36 (D4):	172-175	
VNTR-37 (D3):	194-197	200-203
VNTR-34 (D4):	224-227	260-262
VNTR-19 (D4):	296-299	320-322
VNTR-3 (D2):	392-395	429-431
VNTR-9 (D4):	518-521	566-570

Expected fragment sizes (bp) of the fifteen fragments (locus VNTR_36 is a null allele in EC04PN0139) present in the internal ladder as listed in the peak file:

VNTR-17 (D2):	149-152;	175-178
VNTR-3 (D2):	392-395;	429-431
VNTR-25 (D3):	122-124;	134-136
VNTR-37 (D3):	194-197;	200-203
VNTR-36 (D4):	172-175	
VNTR-34 (D4):	224-227;	260-262
VNTR-19 (D4):	296-299;	320-322
VNTR-9 (D4):	518-521;	566-570

NOTE: fragment sizes for the internal ladder based on independent runs on multiple CEQ instruments at CDC and in PulseNet Participating Laboratories

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