Appendix C

Pathogen Scan Protocol

PCR Pathogen Scan Protocol

A. Sample: Arrival
    1. Samples were shipped on wet ice in Styrofoam containers. At the time of arrival the shipment was checked for integrity and temperature.
    2. Sample logs were checked against the samples to ensure that there were no missing or improperly labeled samples.
    3. The chain of custody form was signed.
    4. The samples were logged into the laboratory's sample logbook, given laboratory ID numbers, and labeled with their laboratory IDs.
    5. Any damaged, leaking, or unacceptable samples were noted.

B. Sample: Short Term Storage
   Samples were stored at four degrees Celsius until they were processed. The samples were processed no longer than four hours after arrival.

C. Sample: Processing
   The samples were processed for bacterial enrichment in liquid media using aseptic technique.
    1. The work surface was wiped with 70% ethanol before and after each sample was processed.
    2. Any scalpels, scissors, or other tools that were used were soaked in ethanol and flamed prior to being used on a sample.
    3. The container that holds the sample was aseptically opened so that the technician does not contaminate the contents.
    4. A portion of the sample was removed using a sterile tool and placed into a labeled enrichment tube. If the sample was a shellfish, it was taken out of the container onto a surface that has been wiped with 70 percent ethanol and cracked open using knives that have been dipped in ethanol and flamed. The shellfish was then cut into portions using a sterile scissors so that it may be inoculated into several broths.
    5. The instruments used to handle the sample were dipped into ethanol and flamed.
    6. The sample container was closed and stored at 4 degrees Celsius until the results of the PCR reactions were obtained. Shellfish were disposed of immediately after processing.

D. Sample: Enrichment
   The bacteria that may be present in a sample were enriched using three liquid enrichment broths. Modified Tryptic Soya broth was used to enrich for gram-negative bacteria. Brain Heat Infusion broth was used to enrich for gram positive and fastidious microorganisms, particularly *Helicobacter pylori* and *Listeria monocytogenes*.

    1. The enrichment media was prepared in 400 milliliter to 800 milliliter volumes according to the manufacturer's directions or published recipes and was steam autoclaved using standard conditions.

2. The autoclaved media was stored at room temperature for several days. Bottles that have any signs of contamination were disposed of.
3. 22.5 milliliters of autoclaved media was aseptically transferred to 50-milliliter screw cap Sarstedt tubes. The caps were screwed on tightly.
4. The Sarstedt tubes were labeled with the laboratory ID of the samples and the date. One uninnoculated blank tube was labeled and prepared in the same way for every ten sample tubes.
5. The sample tubes were inoculated with the 2.5 milliliters or 2.5 grams of the appropriate sample. The caps were left slightly loose.
6. The sample tubes and blank tubes were incubated at 37 degrees and 150 rotations per minute in a shaking incubator overnight. The samples were disposed of if there was any growth in the blank tubes.
7. After incubation was complete, the tubes were placed in a fume hood until further processed. The processing occurs no more than four hours later.

E. Processing of Enrichment Products

The bacterial cells that have grown in the overnight enrichment were lysed for use in DNA amplification reactions. In addition, any cells that may have grown in the enrichment broth were frozen at – 80 degrees Celsius so that they may be recovered at some point in the future

Cell Lysis:

1. 100 microliters of cell suspension was pipetted from each sample enrichment tube and dispensed into a two milliliter tube labeled with the laboratory ID of the sample that contains 800 microliters of lysis buffer. For every fifth sample, a duplicate lysis tube was made.
2. The two milliliter tubes containing sample enrichments and lysis buffer were vortexed, then incubated at 55 degrees Celsius for one hour, followed by 95 degrees Celsius for 10 minutes, and then they were placed on ice or in a – 20 degree Celsius freezer.

Culture Freezing:

1. Following the preparation of the cell lysates, 7.5 milliliters of enrichment was pipetted from the sample enrichment tube and mixed with a labeled 12 milliliter tube containing 2.5 milliliters of freezing media, which consists of 40 % TSB and 60% glycerol.
2. The 12 milliliters tubes containing sample enrichments and freezing media were tightly capped, vortexed, placed in a labeled bag, and then put into a –80 degree Celsius freezer.

F. Amplification of DNA from Lysed Bacterial Cells

Amplification of DNA using primers specific for different strains of bacteria was performed using the Polymerase Chain Reaction (PCR) technique.

1. The theromoclycler was turned on at least 45 minutes prior to the beginning of a PCR reaction program so that it has time to warm up.
2. A cocktail was prepared with primers for the strain of bacteria whose DNA was amplified. The cocktail consists of all the reagents necessary except for the sample DNA and positive control DNA. The cocktail contains enough reagents

for reactions for all of the samples plus: one blank reaction for every seven samples, one positive control reaction for every fourteen samples, and one extra reaction for every ten reactions to be run. The blank reactions ensure that the reaction cocktail, the pipettor, and the pippetor tips were not contaminated with amplifiable DNA. The positive control reactions ensure that the reaction cocktail produces the desired product and allows for comparison between sample reaction products and the reaction product of a true positive.

3. Autoclaved 0.2 ml PCR tube strips were placed into a tube holder. The openings of the tubes were not touched while they were handled.
4. 40 microliters of the reaction cocktail was added to each tube using a pipettor that was only used during the preparation and dispension of the reaction cocktail
5. 10 microliters of sample lysate, ten microliters of autoclaved distilled water, or 1 microliter of control DNA and nine microliters of autoclaved distilled water were added to the appropriate sample, blank, or positive control tube using a pippetor that was only used to load samples, positive control DNA, and water.
6. Autoclaved caps were placed over the openings of the PCR tubes. The inner surfaces were not touched while the caps were handled.
7. The PCR tube holder was placed into the thermocycler and the appropriate program was started after reviewing the program log adjacent to the thermocycler. The technician monitors the thermocycler to ensure that the correct program has started and was running properly.
8. The location of the PCR tube where each sample, blank, and positive control has been loaded was recorded on a log sheet.
9. The PCR reaction products were loaded onto an agarose gel no longer than 24 hours after the reaction was started.

G. Agarose Gel Electrophoresis of DNA Amplification Products

The reaction products from the PCR reactions were separated using agarose gel electrophoresis to determine if bands of the correct sizes have been generated. The bands that samples generate were compared to the positive control and to a DNA size standard.

1. Mix the reagents for a 2 % agarose gel in 0.5 X TBE in a loosely covered Erlenmeyer flask and dissolve/boil the agarose using a microwave or flame. The mixture was shaken several times while being heated. It was removed from the microwave or flame if necessary to prevent overboiling. The mixture was ready to be cooled to approximately 50 degrees Celsius when large bubbles begin to form.
2. While the mixture was cooling, the ends of a gel mold were taped to prevent the mixture from leaking out after it has been poured. The mold was then leveled to ensure that the entire gel was the same thickness. After it has been leveled, combs were set into the gel at the appropriate height and distance from one another.
3. After the gel mixture has cooled to the appropriate temperature, it was poured into the gel mold. Any large bubbles that have formed were removed.
4. As the gel was cooling, 7.5 microliters of loading dye were added to each reaction tube using a pippetor that was only used to add loading dye. The loading dye that was loaded was transferred from the stock bottle to a microcentrifuge tube so that

the stock bottle would not be contaminated with amplification products. A new pippettor tip was used for each sample.

5. After the gel has solidified the combs and the tape on the ends were removed and it was placed into a gel box containing fresh 0.5 X TBE. The surface of the gel was completely covered by the 0.5 X TBE.
6. A gel sheet was prepared according to the following protocol: The size standard was loaded in the left-most lane of the gel. Then, seven sample reactions and one blank reaction were loaded, followed by another seven sample reactions and one blank reaction. The next lane was left empty. The following lane was loaded with the positive control.
7. The gel was loaded according to the gel sheet that has been prepared.
8. The gel was run at 210 volts for 40 minutes.
9. A container containing 200 microliter of 10 mg/mi of ethidium bromide in 200 ml of distilled water was prepared. After the gel was done running it was removed from the gel box and gel mold and placed into the casserole.
10. The gel was stained for thirty minutes, then destained for thirty minutes.
11. Using a UV transilluminator, the bands from the size standard were observed to ensure that the gel has run properly. Next, the bands from the positive control were observed to ensure that the PCR reaction has yielded the desired amplification products. Then, the lanes for the blank reactions were observed to ensure that no undesired reaction products have formed. If all the above conditions have been met, a picture of the gel was taken using a gel documentation system.
12. The results of the sample reactions were interpreted.

H. QA/QC Protocols enforced throughout the process.

1. Gloves were worn whenever handling samples, sample tubes, reaction tubes, and agarose gels.
2. The temperatures of the incubators were checked and written down in a log book before beginning incubation and after incubation was complete. If the temperature was greater than +/- 5 degrees of the desired value, the incubation was repeated with new aliquots of the sample.
3. The pipettors were cleaned on a weekly basis and calibrated on a monthly basis. Pipettors that dispense greater than +/- 5 % of the desired volume were labeled “DO NOT USE” and not used until they have been repaired.
4. All dirty materials, samples, and sample container were decontaminated and disposed of properly.

I. Supplies and Reagents:

Taq Polymerase: from Promega. 5 units per microliter. 1.5 units per reaction.
DNTPs: from Promega, stock solution was 100 millimolar. Working solution was 10 millimolar.
MgCl2: from Promega, 25 millimolar.
10 X magnesium free reaction buffer: from Promega.

Autoclaved deionized water.

10 millimolar Tris: from ICN, prepared from powder and autoclaved deionized water to a concentration of 10 millimolar.
0.5 X TBE
Agarose: from GIBCO
Achromopeptidase: from Sigma, prepared from powder and autoclaved deionized water to a concentration of 5 units per microliter.
Lysis Buffer: 62.5 units of Achromopeptidase per mililiter of 10 millimolar Tris

Autoclaved 1.5 ml tubes.
Autoclaved 2.0 ml tubes.
Autoclaved PCR tube strips
Autoclaved PCR cap strips
Autoclaved pippetor tips

Two 20 micro liter pipettes, one for preparing the PCR reaction, one for post reaction products
Three 200 micro liter pipette, one for preparing the cell lysates, one for preparing the PCR reaction, one for post reaction products
One 1000 micro liter pipette, one for preparing the cell lysates

Gel Mold and Combs
Ethidium Bromide
Gel Box
Incubator
Tube Racks
PCR strip holders
Thermocycler

Recipes for Liquid Enrichment Broths:

Modified Tryptic Soya Broth (mTSB):
30 g Tryptic Soya Broth
1.5 g Bile Salts No. 3
1.5 g Sodium Phosphate, Dibasic
1 milliliter of Novobiocin, at a concentration of 100 mg per ml, after the media has been autoclaved
1 liter of distilled water

Brain Heart Infusion - Difco 0037 (BHI):
Prepare the media per manufacturer's directions.

PCR Reaction Cocktail:
Each PCR reaction takes place in a total volume of 50 microliters consisting of 5 microliters of 10 X Magnesium Free Reaction Buffer (Promega Madison, WI), 4 microliters of 25 millimolar $MgCl_2$ (Promega), one microliter of 10 micromolar working solutions of each of the four DNTPs, and 1.5 units of Promega Taq Polymerase, a volume of primer which yields the desired

reaction product, 10 microliters of sample or 1 microliter of positive control DNA, and a volume of sterile water to bring the reaction volume to 50 microliters.

PCR Primers and Thermal Conditions:

Primer sequences and thermal conditions for gene targets from pathogenic bacteria.			
Organism (Reference)	Amplification Target (size, base pairs)	Forward Primer Reverse Primer	Thermal Conditions (Number of cycles)
Esherichia coli O157	*eae* gene (384)	5'-GACCCGGCACAAGCATAAGC-3' 5'-CCACCTGCAGCAACAAGAGG-3'	95 C/ 1m – 65 C/ 2m – 72 C/ 90s (10) 95 C/ 1m – 65>60 C/ 2m – 72 C/ 90s (5) 95 C/ 1m – 60 C/ 2m – 72 C/ 90s (10) 95 C/ 1m – 60 C/ 2m – 72 C/1.5>2m (10)
	hlyA gene (534)	5'-GCATCATCAAGCGTACGTTCC-3' 5'-AATGAGCCAAGCTGGTTAAGCT-3'	
	stx1 gene (180)	5'-ATAAATCGCCATTCGTTGACTAC-3' 5'-AGAACGCCCACTGAGATCATC-3'	
	stx2 gene (255)	5'-GGCACTGTCTGAAACTGCTCC-3' 5'-TCGCCAGTTATCTGACATTCTG-3'	
Listeria spp. and *Listeria monocytogenes*	Eubacteria 16s rRNA (408)	5'- CAG CMG CCG CGG TAA TWC-3' 5'-CCG TCA ATT CMT TTR AGT TT-3'	94 C/ 80 s – 50 C/ 90 s – 72 C/ 2 m (24) 72 C/ 10 m
	Listeria spp. 16s rRNA (938)	5'- CAG CMG CCG CGG TAA TWC-3' 5'- CTC CAT AAA GGT GAC CCT-3'	
	Listeria monocytogenes listeriolysin O (702)	5'- CCT AAG ACG CCA ATC GAA-3' 5'- AAG CGC TTG CAA CTG CTC-3'	
Salmonella spp.	Chromosome (429)	5'- AGC CAA CCA TTG CTA AAT TGG CGC A -3' 5'- TTT GCG ACT ATC AGG TTA CCG TGG -3'	94 C/ 1 m – 53 C/ 2 m – 72 C/ 3 m (35)
Helicobacter pylori	Urease gene A (411)	5'-GCCAATGGTAAATTAGTT-3' 5'-CTCCTTAATTGTTTTTAC-3'	95 C/ 5 m 94 C/ 1m – 45 C/ 1m – 72 C/ 1m (35) 72 C/ 4 m
Salmonella spp.	Chromosome (429)	5'- AGC CAA CCA TTG CTA AAT TGG CGC A -3' 5'- TTT GCG ACT ATC AGG TTA CCG TGG -3'	94 C/ 1 m – 53 C/ 2 m – 72 C/ 3 m (35)
Staphylococcus aureus	Nuclease gene (276)	5'- GCGATTGATGGTGATACGGTT-3' 5'-CAAGCCTTGACGAACTAAAGC-3'	94 C/3 s - 58 C/10 s - 72 C/35 s; 72 C/2 min
Vibrio parahemolyticus	*toxR* gene (~400)	5'-GTCTTCTGACGCAATCGTTG-3' 5'-ATACGAGTGGTTGCTGTCATG-3'	94 C/ 1 m – 63 C/ 90s – 72 C/ 90s (20)
Vibrio vulnificus	Cytolysin gene (383)	5'- CTCACTGGGGCAGTGGCT-3' 5'-CCAGCCGTTAACCGAACCA-3'	94 C/3 s - 58 C/10 s - 72 C/35 s; 72 C/2 min (35)