6.0 Analytical Procedures

Analytical procedures performed by Weston Solutions, Inc. are summarized in this section. Standard Operating Procedures (SOPs) pertaining to these methods are found in the SOP manual.

SOPs are dated and revisions are numbered and dated. The revision date reflects the date that the SOP was first put into effect. When a SOP is revised, the earlier version is filed, and the end date of effect is noted on the filed SOP. Thus, the time period during which the protocol was in place is documented.

Standard Operating Procedures and Testing Protocols for the laboratories are updated by the quality assurance officer as needed and reviewed at least biannually. If there are no changes, the existing SOP may be resigned and dated. Hand written edits, accompanied by initials and date, are acceptable for minor updates until such time as the document is reissued and assigned a new revision number.

Procedures for which Weston Solutions, Inc. is accredited are found in the latest ELAP issued Fields of Testing Summary (Certification number 2613). The most current listing of approved fields of testing can be found by customers on the State of California web site:

http://www.dhs.ca.gov/ps/ls/elap/ELAPnames/Laboratory_37.htm); and

Microbiological organisms in water samples are detected and enumerated using membrane filtration, multiple tube fermentation or IDEXX chromogenic substrate procedures.

6.1 Membrane Filtration

For the analysis of total and fecal coliforms, enterococci and fecal streptococci in non-turbid waters.

6.1.1 Total Coliforms

For analysis of total coliforms, petri dishes are prepared using M-Endo LES agar. The bottoms of petri dishes are labeled with sample identification, volume of sample filtered, date, and technician's initials. The filter manifold is flame-sterilized, and allowed to cool; and a sterile filter is placed onto the manifold. The sample is well mixed and then pipetted onto the filter. The vacuum is turned on, and the sample is allowed to filter through the membrane. After filtering is complete, the filter is removed from the manifold and placed onto the prepared media. Plates are inverted and incubated at 35 $\pm$ 0.5°C for 22 to 24 hours. Coliform colonies (pink to dark red with a metallic surface sheen) are counted, and recorded.

Only plates with 20 to 80 coliform colonies and not more than 200 colonies of all types are counted. Plates are counted as follows:

$$\text{Total coliform colonies / 100 mL} = \frac{\text{Coliform colonies counted x 100}}{\text{mL sample filtered}}$$

6.1.2 Fecal Coliforms

For analysis of fecal coliforms, petri dishes are prepared using M-FC agar. The bottoms of petri dishes are labeled with sample identification, volume of sample filtered, date, and technician's initials. The filter manifold is flame-sterilized, and allowed to cool; and a sterile filter is placed onto the manifold. The sample is well mixed and then pipetted onto the filter. The vacuum is turned on, and the sample is allowed to filter through the membrane. After filtering is complete, the filter is removed from the manifold and placed onto the prepared media. Plates are inverted and incubated in a waterbath at 44.5 ± 0.2°C for 24 ± 2 hours. Fecal coliform colonies (various shades of blue) are counted, and recorded.

Only plates with 20 to 60 coliform colonies and not more than 200 colonies of all types are counted. Plates are counted as follows:

$$\text{Fecal coliform colonies / 100 mL} = \frac{\text{Fecal coliform colonies counted x 100}}{\text{mL sample filtered}}$$

6.1.3 Enterococci

For analysis of enterococci in non-turbid water, petri plates are prepared using mE agar and EIA substrate. Plates of both types are kept covered at all times due to light sensitivity, and are refrigerated until shortly before use.

The bottoms of mE agar petri dishes are labeled with sample identification, volume of sample filtered, date, and technician's initials. The filter manifold is flame-sterilized, and allowed to cool, and a sterile filter membrane is placed onto the manifold. The sample is well mixed and pipetted onto the filter. The vacuum is turned on, and the sample is allowed to filter through the membrane. After filtering is complete, the membrane is removed from the manifold and placed onto the prepared media. Within 30 minutes maximum of filtration, plates are inverted and placed in a 41°C ± 0.5°C incubator for 48 hours. Upon completion of incubation, membranes with colonies are carefully transferred to EIA plates with corresponding identification written on bottom of plates. EIA plates are incubated at 41°C ± 0.5°C for 20 minutes. As pink to red enterococci colonies hydrolyze esculin, they will develop a black or reddish-brown precipitate on the underside of the filter.

Only plates with 20 to 60 coliform colonies and not more than 200 colonies of all types are counted. Plates are counted as follows:

$$\text{Enterococci / 100 mL} = \frac{\text{Enterococci colonies counted x 100}}{\text{mL sample filtered}}$$

6.1.4 Verification

On a monthly basis or when procedure is performed, at least ten isolated, typical enterococci colonies are picked from membranes on EIA media and are streaked for isolation onto brain-heart infusion agar plates. Plates are incubated at 35°C ± 0.5°C for 24-48 hours. Upon completion of incubation, growth from a well-isolated colony is transferred, using a sterile

inoculating loop, into a brain-heart infusion (BHI) broth tube and to each of two clean glass slides. The tubes are incubated at 35°C ± 0.5°C for 24 hours.

Simultaneously, a few drops of freshly prepared 3% hydrogen peroxide are added to one slide. The presence of bubbles indicates the colony is not a member of the fecal streptococcus group. If the catalase test is negative (no bubbles), a Gram stain is performed on the second slide. Enterococci are defined as Gram-positive, ovoid cells, 0.5 to 1.0 µm in diameter, mainly in pairs or short chains.

Upon incubation of each of the BHI tubes, a loopful of growth is transferred from the tubes to each of the following media: brain-heart infusion broth (incubate at 45°C ± 0.5°C for 48 hours) and brain-heart infusion broth with 6.5% NaCl (incubate at 35°C ± 0.5°C for 48 hours). Growth of catalase-negative, Gram positive cocci at 45°C and in 6.5% NaCl broth indicates the colony belongs to the enterococcus group. Results are observed and recorded.

6.1.5 Fecal Streptococci

For analysis of fecal streptococci in non-turbid waters, petri dishes are prepared using m-Enterococcus agar. Plates are kept covered at all times due to light sensitivity, and are refrigerated until shortly before use. The bottoms of petri dishes are labeled with sample identification, volume of sample filtered, date, and technician's initials. The filter manifold is flame-sterilized, and allowed to cool; and a sterile filter is placed onto the manifold. The sample is well mixed and pipetted onto the filter. The vacuum is turned on, and the sample is allowed to filter through the membrane. After filtering is complete, the filter is removed from the manifold and placed onto the prepared media. Plates are allowed to stand for 30 minutes after filtration, and are then inverted and placed in a 35°C ± 0.5°C incubator for 48 hours. All light and dark red colonies are counted as positive for fecal streptococcus. Results are observed and recorded.

Only plates with 20 to 60 coliform colonies and not more than 200 colonies of all types are counted. Plates are counted as follows:

$$\text{Fecal streptococci colonies / 100 mL} = \frac{\text{Fecal streptococci colonies counted x 100}}{\text{mL sample filtered}}$$

6.1.6 Verification

On a monthly basis or when procedure is performed, at least ten isolated, typical fecal streptococci colonies are picked from membranes on m-Enterococcus media and are streaked for isolation onto brain-heart infusion agar plates. Plates are incubated at 35°C ± 0.5°C for 24-48 hours. Upon completion of incubation, growth from a well-isolated colony then transferred, using a sterile inoculating loop, into a brain-heart infusion (BHI) broth tube and to each of two clean glass slides. The tubes are incubated at 35°C ± 0.5°C for 24 hours.

Simultaneously, a few drops of freshly prepared 3% hydrogen peroxide are added to one slide. The presence of bubbles indicates the colony is not a member of the fecal streptococcus group. If the catalase test is negative (no bubbles), a Gram stain is performed on the second slide. Fecal streptococci are defined as Gram-positive, ovoid cells that are 0.5 to 1.0 µm in diameter, mainly in pairs or short chains.

Upon incubation of each of the BHI tubes, a loopful of growth is transferred from the tubes to each of the following media: bile esculin agar (incubate at 35°C ± 0.5°C for 48 hours) and brain-heart infusion broth (incubate at 45°C ± 0.5°C for 48 hours). Growth of catalase-negative, Gram positive cocci on bile esculin agar and at 45°C in brain-heart infusion broth verifies that the colony is of the fecal streptococcus group. Results are observed and recorded.

6.2 Multiple Tube Procedures

This procedure is used to test for total and fecal coliforms, fecal streptococcus and enterococcus and may be used with turbid waters, sediments or tissue.

6.2.1 Total and Fecal Coliform Presumptive Test for Wastewater, Recreational Water and Shellfish Growing Waters

Pre-filled LTB test tubes with inverted Durham tubes are arranged in rows of five, with the number of rows corresponding to the number of straight sample and/or its dilutions being tested. Samples are well mixed and each tube in the five-tube row is uncapped, inoculated with replicate sample volumes, and then recapped. Upon inoculation, samples are incubated at 35°C ± 0.5°C. After 24 and 48, samples are observed for presence of air bubbles in the inverted tubes. Tubes showing turbidity and gas formation provide a positive result for the presumptive portion of the test. Results are recorded on laboratory bench sheets, and all tubes showing positive results are subjected to the Confirmed Test.

6.2.2 Total and Fecal Coliform Presumptive Test for Shellstock

A minimum of 12 shellfish (or more) are collected to obtain a representative sample, from which an aliquot of approximately 200g is used (if 12 animals provide more than 200gr of shellfish meat and liquor, the sample is always blended first and than 200gr are removed for testing). Properly cleaned shellfish and accompanying liquor are transferred to a sterile, tared, blender jar and weighed to the nearest 0.1 gram. An equal amount by weight of phosphate buffered dilution water is added to the sample and blended up to 90 sec.

6.2.2.1 Presumptive Test Using Multiple Dilutions

The ground sample is cultured within two minutes after the completion of the grinding period. The sample is thoroughly mixed and transferred to LTB fermentation tubes in the following manner.

Each set of five tubes are inoculated with 2 mL of ground sample, 1 mL of a 1:10 dilution of the shellfish sample, 1 mL of a 1:100 dilution of the shellfish sample, 1 mL of a 1:1,000 dilution of shellfish sample, and 1mL of 1:10,000 dilution of shellfish sample. However, to attain assurance with samples of doubtful quality it may be necessary to inoculate additional decimal dilutions.

Tubes and/or racks are labeled carefully according to date and sample identification. Prior to pipetting all samples are well mixed. Each tube in the five-tube row is uncapped one at a time, inoculated with replicate sample volumes, and recapped. Within 20 minutes maximum of inoculation, inoculated samples are incubated at 35°C ± 0.5°C for 24 ± 2 hours. Upon

completion of incubation, each tube is observed for turbidity and presence of air bubbles in inverted tube. All results are recorded as specified on worksheet and all tubes presenting as positive after 24 hours are subjected to the Confirmed Phase.

6.2.2.2 12-Tube Single Dilution Presumptive Test (End product shellfish samples only)

The ground sample is cultured within two minutes after the completion of the grinding period. The sample is thoroughly mixed and transferred to LTB fermentation tubes.

Pre-filled LTB test tubes with inverted Durham tubes are arranged in three rows of four. Using a single strength LTB broth each set of four tubes is inoculated with 2 mL of ground sample. Each tube in the four-tube row is uncapped one at the time, inoculated with replicate sample volumes, and then recapped. Tubes and/or racks are labeled carefully according to date and sample identification and preparer's initials. Completed test tube racks are incubated at 35°C ± 0.5°C for 24 ± 2 hours.

Upon completion of incubation, each tube is observed for presence of air bubbles in inverted tubes. Tubes showing gas formation provide a positive result for the presumptive portion of the test. Positive and negative results are recorded as specified on laboratory bench sheet and all tubes showing positive results after 24 hours are subjected to the Confirmed Test for fecal coliforms.

6.2.3 Total and Fecal Coliform Confirmed Tests

6.2.3.1 Total Coliform Confirmed Test for Wastewater, Recreational Water and Shellfish Growing Waters

Samples from each positive presumptive phase tube are aseptically transferred into a coliform selective BGB fermentation tube using a sterile inoculating loop. All tubes are labeled accordingly. Upon inoculation of new BGB tubes, tubes with positive results from the presumptive test are recorded and then discarded and replaced with their corresponding inoculated BGB tubes. Racks with new BGB tubes and 24-hour negative LTB tubes are placed back in incubator and incubated at 35°C ± 0.5°C for 24 ± 2 hours.

Upon completion of incubation, each 24-hour BGB and 48-hour LTB tube is observed for presence of air bubbles in inverted tube. BGB tubes showing gas formation and turbidity provide a positive result for the confirmed portion of the test. Positive and negative results are recorded as specified on laboratory bench sheets.

All negative BGB tubes are placed back in incubator for an additional 24 hours (total incubation time of 48 ± 3 hours), and any additional positive results are noted. Total coliform MPN values are calculated from the total number of positive BGB tubes and recorded.

6.2.3.2 Total Coliform Confirmed Test for Shellfish

Samples from each positive presumptive phase tube are aseptically transferred into a coliform selective BGB fermentation tube using a sterile inoculating loop. All tubes are labeled accordingly. Upon inoculation of new BGB tubes, tubes with positive results from the presumptive test are recorded and then discarded and replaced with their corresponding inoculated BGB tubes. Racks with new BGB tubes are placed back in incubator and incubated at 35°C ± 0.5°C for 24 ± 2 hours.

Upon completion of incubation, each 24-hour BGB tube is observed for presence of air bubbles in inverted tube. BGB tubes showing gas formation and turbidity provide a positive result for the confirmed portion of the test. Positive and negative results are recorded as specified on laboratory bench sheets.

Total coliform MPN values are calculated from the total number of positive BGB tubes and recorded.

6.2.3.3 Fecal Coliform Confirmation Test for Wastewater, Recreational Water, Shellfish Growing Waters and Shellfish

Simultaneously with the total coliform confirmation (BGB) test being performed; samples from each positive presumptive phase LTB tube are aseptically transferred into a fecal coliform selective EC fermentation tube using a sterile inoculating loop. All tubes are labeled accordingly. Within 20 minutes of inoculation, tubes are placed in well labeled racks in a waterbath and incubated at 44.5°C ± 0.2°C for 24 ± 2 hours.

Upon completion of incubation, each tube is observed. Tubes showing turbidity and any gas formation in inverted tubes provide a positive confirmation for fecal coliform bacteria. Positive and negative results are recorded as specified. Fecal coliform MPN values are calculated from the total number of positive EC tubes and recorded as MPN/100mL for all water samples and MPN/100g for shellfish.

6.2.4 Total and Fecal Coliform Completed Test

This step is not required in samples analyzed in the depurated shellfish program. This portion of the procedure applies to wastewater, recreational water and non depurated shellfish only.

For quality control and definitive confirmation, the completed test is performed seasonally on a specified day on ≥10% of all tubes testing positive in the confirmed phase. In any case, a minimum of one positive BGB and one EC tube is tested every three months.

Using a sterile loop, M-Endo LES agar plates are aseptically streaked with samples from desired positive BGB and EC tubes. Plates should be streaked so as to provide isolated colonies separated by at least 0.5 cm. The bottoms of plates are labeled and plates are inverted and incubated at 35°C ± 0.5°C for 24 ± 2 hours. Upon completion of incubation, typical coliform colonies appear pink to dark red with a green metallic sheen. Atypical colonies are colorless and translucent.

From each M-Endo plate, the following is performed:

- One typical, well-isolated colony is carefully picked and cells aseptically transferred to an LTB fermentation tube. (If no typical colonies are present, one that is most likely to be a positive coliform is chosen.) Tubes are incubated at 35°C ± 0.5°C for 24 ± 2 hours.
- Upon completion of incubation, secondary LTB tubes are observed for turbidity and gas, and results are recorded. If no gas is present, tubes are re-incubated and observed again at 48 ± 3 hours. Gas formation after 48 ± 3 hours indicates a positive result, while no gas indicates a negative result. Results are recorded and maintained.

6.2.4.1 Fecal Coliform Multiple Tube Fermentation Direct Test Method for Wastewater, Recreational Water and Shellfish Growing Waters

Pre-filled A-1 tubes (media must be made from individual ingredients for shellfish growing water samples) with inverted Durham tubes are arranged in rows of five, with the number of rows corresponding to the number of straight sample and/or its dilutions being tested. Sets of five tubes are inoculated with each sample portion, i.e., five tubes of 10 mL, five tubes of 1 mL, and five tubes of 0.1 mL. However dilutions and the number of five tube rows are determined by the quality of water to be examined, and additional dilutions may be required depending on bacterial concentration.

Tubes and/or racks are labeled carefully according to date and sample identification. Prior to pipetting all samples are well mixed. Each tube in the five-tube row is uncapped one at a time, inoculated with replicate sample volumes, and then recapped. Tubes and/or racks are labeled carefully according to date and sample identification. Completed test tube racks are placed in air incubator with humidity for three ± 0.5 hours for shellfish growing waters and three hours for wastewater and recreational waters at 35°C ± 0.5°C. Samples are then transferred to a waterbath and incubated at 44.5°C ± 0.2°C for an additional 21 ± 2 hours.

After incubation is complete, tubes are observed for turbidity and for presence of air bubbles in inverted tubes. Gas formation in any A-1 broth culture tube within 24 hours or less is confirmed positive for fecal coliform bacteria. Results are recorded on laboratory bench sheets

Using the Standard Methods MPN Table (APHA Table for shellfish growing waters), values are calculated from the total number of positive A-1 tubes and recorded in MPN/100mL.

6.2.5 Presence-Absence Coliform Test for UV Treated Depuration Process Water

Pre-filled double strength LTB test tubes are arranged in one row of ten tubes and labeled with sample identification, date and preparer's initials. Samples are well mixed and each tube in the ten-tube row is uncapped, inoculated with 10mL of straight sample, and then recapped. Within 20 minutes of inoculation, samples are incubated at 35°C ± 0.5°C. After 24 and 48 hours, samples are observed and any tubes showing turbidity provide a positive, and thus failing, result for the test. Results are recorded on laboratory bench sheets and are reported as pass/fail.

6.2.6 Fecal Streptococcus and Enterococcus Presumptive Test

Prepared azide tubes are arranged in rows of five, with the number of rows corresponding to the number of straight sample and/or its dilutions being tested. Dilutions and the number of five tube rows are determined by the quality of water to be examined, and adjustments may be required depending on bacterial concentration. Samples are vortexed; each tube in the five-tube row is uncapped, inoculated with replicate sample volumes, and recapped. Upon inoculation, each tube is gently swirled to allow distribution of sample, while prohibiting the contents from spilling. Completed test tube racks are labeled carefully according to date and sample identification, and placed in an incubator at 35°C ± 0.5°C. Upon completion of 24 ± 2 hours of incubation, tubes are observed for turbidity. If no definite turbidity is present, tubes are re-incubated and read again at the end of 48 ± 3 hours total. Positive and negative results are recorded, and all tubes showing turbidity after 24-48 hours are submitted to the Confirmed Test.

6.2.7 Fecal Streptococcus Confirmed Test

A portion of growth from each positive azide dextrose tube is streaked onto a E agar plate using a sterile inoculating loop, with up to five tubes inoculated per plate. Plates are labeled accordingly and are incubated at 35°C ± 0.5°C for 24 ± 2 hours. Brownish-black colonies with brown halos confirm the presence of fecal streptococci. The number of positive tubes indicated by the brown halos is subjected to the MPN calculation and results recorded.

6.2.8 Enterococcus Confirmed Test

For the enumeration of enterococci, fecal streptococci colonies from above are then transferred to two tubes. One tube of brain-heart infusion (BHI) broth containing 6.5% NaCl, and one tube containing BHI broth only. The BHI broth tubes are placed in a waterbath at 45°C ± 0.5°C for 45 to 48 hours. The BHI + NaCl tubes are placed in an incubator at 35°C ± 0.5°C for 45 to 48 hours. Growth in each of the sample's two corresponding tubes is positive confirmation for enterococci. The number of corresponding matching tubes is used to calculate the MPN, using the Standard Methods MPN Table.

6.2.9 Calculation for Wastewater and Recreational Water Samples

To calculate the MPN, the number of positive tubes in the confirmed tests are used and compared to Standard Methods Table 9221.IV (SM 9221C). Calculations for total coliforms (BGB tubes), fecal coliforms (EC tubes), fecal streptococcus (E agar plates) and enterococci (paired BHI and BHI + NaCl tubes) are performed separately.

Values in the table are based on 10, 1, and 0.1mL dilutions. If more than three dilutions are used during testing, only the results from three of these are used. To determine the three dilutions to be used, the highest dilution and corresponding volumes that give positive results in all five portions tested (no lower dilution giving any negative results) and the two next succeeding higher dilutions are used. When the series of decimal dilutions is different from that in the table, the MPN is determined by multiplying the result by the appropriate dilution factor. That is, if sample portions of 1, 0.1, and 0.01 mL are used (vs. 10, 1, 0.1), the MPN result is multiplied by a factor of 10.

6.2.10 Calculation for Shellstock and Shellfish Growing Waters

To calculate the MPN for shellfish growing waters and shellfish fecal coliforms using a multiple tube dilution, the number of positive tubes in the confirmed tests are used and compared to Recommended Procedures 4th ed., 1970, Table 11. Calculations for total coliforms (BGB tubes), fecal coliforms (EC tubes).

Values in the table are based on 10, 1 and 0.1g dilutions. If more than three dilutions are used during testing, only the results from three of these are used. To determine the three dilutions to be used, the highest dilution and corresponding volumes that give positive results in all five portions tested (no lower dilution giving any negative results) and the two next succeeding higher dilutions are used.

When the series of decimal dilutions is different from that in Table 11, the MPN is determined by multiplying the result by the appropriate dilution factor. That is, if sample portions of 1, 0.1, and 0.01 g are used (vs. 10, 1, 0.1 g), the MPN result is multiplied by a factor of 10.

To calculate the MPN for shellfish fecal coliforms using the 12 tube single dilution, the number of positive tubes in the confirmed test is used and compared to National Shellfish Sanitation Program, FDA, Manual of Interpretations, Interpretation number: 01-XV-03-100, Revised December 8, 2002.

The fecal coliform density in shellfish is expressed as MPN per 100g of sample.

6.3 Quality Control/Assurance

Duplicates are performed on ≥ 10% of all samples and results recorded (not required under Shellfish Sanitation Program).

For quality control and definitive confirmation, the Multiple Tube Fermentation, Total and Fecal Coliform Completed Test is performed seasonally on a specified day on ≥10% of all tubes testing positive in the confirmed phase. In any case, a minimum of one positive BGB and one EC tube is tested every three months. This step is not required for shellstock or shellfish growing waters if performed on samples from a shellfish depuration plant.

Positive and negative growth performance tests are performed on each batch of media. This is done for total coliform media by inoculating one tube from each batch with a positive control of *E. coli* and another tube from each batch with a negative control of *Staphylococcus aureus*. This is done for fecal coliform media by inoculating one tube from each batch with a positive control of *E. coli* and another tube from each batch with a negative control of *E. aerogenes*.

Enterococcus and fecal streptococcus media is tested using *E. faecalis* as a positive control and *Staphylococcus aureus or E. coli* as a negative control. In addition, three uninoculated tubes or plates from each batch of media are incubated to confirm media sterility.

To confirm sterility of dilution water/vials if used, a set of negatives is made from the dilution water, as if it had been inoculated. All control tubes are incubated at 35°C ± 0.5°C and checked for gas formation or turbidity after 24 ± 2 and 48 ± 3 hours, and results are recorded.

When the possibility of overnight loss of power exists, the following is performed: To check for proper functioning of waterbaths overnight, two tubes of EC broth with *E. coli* and two tubes of EC broth with *Enterobacter aerogenes* are made. One tube of each is placed in the waterbath at 44°C ± 0.2°C and one of each in an incubator at 35°C ± 0.5°C. *E. coli* should produce turbidity and gas at both temperatures, while *E. aerogenes* should produce turbidity only at 35°C. Lack of turbidity in the *E. aerogenes* tube in the waterbath therefore confirms stability of the 44°C temperature. A blank tube of EC broth is also placed in the waterbath to confirm broth sterility.

For shellfish Positive and Negative Process Controls are performed.

Positive and negative control cultures accompany the sample (or sample set) throughout the procedure, and records are maintained.

For the positive control, prior to receiving a sample, a dilution is performed in the laboratory by inoculating a colony of a pure culture of *E. coli* in 100mL of sterile phosphate buffered saline. Control culture enumeration is then performed using a laboratory-certified method such as Colilert-18 or multiple tube fermentation to derive a count per 1mL of *E. coli*. During sampling, a dilution is performed using sterile phosphate buffered saline in order to derive a cell count of approximately 20 to 30 cells per 1mL. One milliliter of this final solution is added to one test tube of LTB. The test tube is incubated on the rack with the prepared sample(s) and counted after 24 hours. A loopfull of bacteria from the positive control tube is then aseptically transferred to EC along with the positive tubes in the accompanying sample(s). The EC tube is incubated at 44.5ºC ± 0.2ºC for 24 hours and results recorded.

For the negative control, *Staphylococcus aureus* is used by transferring a sterile loopfull of a pure culture directly to an LTB test tube. This tube is also incubated on the rack with the sample(s) being analyzed. All results are recorded.

For shellfish and shellfish growing waters microbiological quality and density of air is determined monthly, by exposing a plate of non-selective agar (such as nutrient agar) to the air for 15 minutes. Exposed plates are incubated overnight. Acceptable plates should have counts of less than 15 colonies.

6.4 IDEXX Chromogenic Substrate Procedure

This procedure is used for the enumeration of total coliforms, *E. coli*, and enterococcus.

6.4.1 IDEXX Chromogenic Substrate Procedure for Total Coliforms and E. coli

For the enumeration of total coliforms and *E. coli*, 100 mL of sample is allowed to reach room temperature. One packet of Colilert, Colilert 18 or Colisure reagent is aseptically added to the sample and shaken vigorously or vortexed until reagent dissolves. If dilutions are performed, the reagent is added to sterile deionized water first (to act as a buffer) and shaken until dissolved. The sample is added last, and shaken or vortexed until completely mixed. The quantitray is pinched/pushed in from either side to open it and the entire sample with reagent is aseptically poured in. A rubber sealer pad is placed facing the front of the quantitray so that all the holes in the pad match the wells in the tray and tray and pad are fed into the sealer. When the tray and sealer pad exit the back of the sealer, the tray is separated from the sealer pad and placed in an incubator with the appropriate temperature and incubation time as follows:

35°C ± 0.5°C for 24-28 hours for Colilert or Colisure
35°C ± 0.5°C for 18-22 hours for Colilert 18

Upon completion of incubation, the quantitray is counted for total coliforms and *E. coli*. For Colilert and Colilert 18, yellow wells enumerate total coliforms. For Colisure, magenta wells enumerate coliforms. All large and small yellow/magenta wells are counted and results inserted into the IDEXX - MPN program or are interpolated from the IDEXX - MPN chart. Using a

365λ fluorescent lamp, the quantitray is then illuminated, and all large and small wells are checked for fluorescence in the same way as the wells for total coliforms. The fluorescent wells enumerate *E. coli* for all methods. Results are recorded and reported as MPN/100 mL.

6.4.2 IDEXX Chromogenic Substrate Procedure for Enterococci

For the enumeration of enterococci, 100 mL of sample is allowed to reach room temperature. One packet of Enterolert reagent is aseptically added to the sample and shaken vigorously or vortexed until reagent dissolves. If dilutions are performed, the reagent is added to sterile deionized water first and shaken until dissolved. The sample is added last, and shaken or vortexed until completely mixed. The quantitray is pinched/pushed in from either side to open it and the entire sample with reagent is aseptically poured in. A rubber sealer pad is placed facing the front of the quantitray so that all the holes in the pad match the wells in the tray and the tray with pad are fed into the sealer. When the tray and pad exit the back of the sealer, the tray is separated from the sealer pad and placed in an incubator at 41°C ± 0.5°C for 24-28 hours. Upon completion of incubation, the quantitray is counted for enterococci by using a 365λ fluorescent lamp to check for fluorescence. The numbers of large and small fluorescent wells are counted and the results inserted into the IDEXX - MPN program or are interpolated from the IDEXX - MPN chart. Results are recorded and reported as MPN/100 mL.

6.4.3 IDEXX Chromogenic Procedure for Heterotrophic Plate Counts

This method is used for the enumeration of live heterotrophic bacteria in laboratory's in house e-pure water. Sample is collected from e-pure system. Tube containing media is uncapped and 10mL ± 0.2mL of sample is added. Media tube is re-capped and shaken ≥ 25 times to ensure proper dispersion of cells. Contents of the tube containing media are aseptically poured onto the center of the plate base. The plate is covered with the lid and gently swirled to distribute the sample into all wells. The plate is then inverted and placed in the incubator for 48 hours at 35^{o}C ± 0.5^{o}C. Upon completion of incubation, plate is removed from the incubator, uncovered and the number of wells showing fluorescence is counted under a 365λ UV light.

All wells showing fluorescence are recorded and results inserted into the IDEXX - MPN chart. Results are reported as MPN/1mL.

6.5 Water Supply

Water used in the preparation of media solutions and buffers is E-pure water from System II, described in Table 6.1. System II water is filtered through a 0.2-µm filter to remove bacterial contamination.

Water quality is monitored continuously for conductivity. A continuously-lit LED indicates that the water has a minimum resistance of 1 mega-ohm. Records are maintained for all water quality monitoring.

Table 6.1: Quality of Water Used in Media Preparation

Test	Monitoring Frequency	Limit
Chemical tests:		
Heavy metals, single (Cd, Cr, Cu, Ni, Pb, and Zn)	Annually*	< 0.05 mg/L
Heavy metals, total	Annually*	≤ 0.1 mg/L
Conductivity	Monthly	< 2 micromhos/cm resist.
Heterotrophic plate count	Monthly	< 500 CFU/mL
Total chlorine residual	Monthly	< <0.1 mg/L (DPD Method)
Ammonia/organic nitrogen	Monthly	<0.1 mg/L
Water suitability test	Annually	0.8-3.0 ratio
Inhibitory Residue Test	Annually	Pass/Fail

*Or more frequently if there is a problem