Total Maximum Daily Load Grant Program

Bacterial Source Tracking for Little Brazos River Tributaries Bacteria Assessment

TSSWCB Project 09-52
Revision 0

Quality Assurance Project Plan

Texas State Soil and Water Conservation Board

prepared by

Texas AgriLife Research
Texas Water Resources Institute

Effective Period: Upon Approval through May 2010

Questions concerning this quality assurance project plan should be directed to:

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A1 APPROVAL PAGE

Quality Assurance Project Plan for *Bacterial Source Tracking for Little Brazos River Tributaries Bacteria Assessment.*

Texas State Soil and Water Conservation Board

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A2 TABLE OF CONTENTS

A3 DISTRIBUTION LIST

Organizations, and individuals within, which will receive copies of the approved QAPP and any subsequent revisions include:

Texas State Soil and Water Conservation Board
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Name: Jay Bragg
Title: Central/Lower Regional Environmental Planner

Name: Tiffany Morgan
Title: Environmental Services Manager

List of Acronyms

ACS	American Chemical Society
EP AREC	El Paso AgriLife Research and Extension Center
BAEN	TAMU Biological & Agricultural Engineering Department
BRA	Brazos River Authority
BST	bacterial source tracking
CAR	corrective action report
COC	chain of custody
DNA	deoxyribonucleic acid
DQO	data quality objective
EPA	U.S. Environmental Protection Agency
ERIC-PCR	enterobacterial repetitive intergenic consensus PCR
ERIC-RP	ERIC-PCR / RiboPrinting combination method
mi^2	square miles
mRNA	messenger ribonucleic acid
MUG	methylumbelliferyl-β-D-glucuronide
NA-MUG	nutrient agar with MUG
NELAC	National Environmental Laboratory Accreditation Conference
NIST	National Institute of Standards and Technology
PCR	polymerase chain reaction
PM	project manager
QA	quality assurance
QC	quality control
QAO	Quality Assurance Officer
QAPP	quality assurance project plan
QPR	quarterly progress report
RP	RiboPrinting
RPD	relative percent difference
rRNA	ribosomal ribonucleic acid
SAML	Soil and Aquatic Microbiology Laboratory at AgriLife Research SCSC
SCSC	Soil and Crop Science Department, Texas AgriLife Research
SM	Standard Methods for Examination of Water and Wastewater, 21^{st} edition
SOP	standard operating procedure
ST	total samples
SV	valid samples
SWQM	surface water quality monitoring
tRNA	transfer ribonucleic acid
TAMU	Texas A&M University
TCEQ	Texas Commission on Environmental Quality
TSSWCB	Texas State Soil and Water Conservation Board
TWRI	Texas Water Resources Institute
UV	ultraviolet light

A4 PROJECT/TASK ORGANIZATION

The following is a list of individuals and organizations participating in the project with their specific roles and responsibilities:

Texas State Soil and Water Conservation Board

Loren Henley, TSSWCB Project Manager

Responsible for ensuring that the project delivers data of known quality, quantity, and type on schedule to achieve project objectives. Provides the primary point of contact between the TWRI and the TSSWCB. Tracks and reviews deliverables to ensure that tasks in the work plan are completed as specified in the contract. Notifies the TSSWCB QAO of significant project nonconformances and corrective actions taken as documented in QPRs from TWRI PM.

Donna Long, TSSWCB Quality Assurance Officer

Reviews and approves QAPP and any amendments or revisions and ensures distribution of approved/revised QAPPs to TSSWCB participants. Responsible for verifying that the QAPP is followed by the TWRI. Assists the TSSWCB PM on QA-related issues. Coordinates reviews and approvals of QAPPs and amendments or revisions. Conveys QA problems to appropriate TSSWCB management. Monitors implementation of corrective actions. Coordinates and conducts audits.

Texas AgriLife Research, Texas Water Resources Institute

B.L. Harris, TWRI Associate Director and Project Co-Lead

Responsible for coordinating attendance at conference calls, training, meetings, and related project activities with the TSSWCB. Responsible for verifying that the QAPP is distributed and followed by all participants, and that the project is producing data of known and acceptable quality. Responsible for the facilitation of audits and the implementation, documentation, verification and reporting of corrective actions. Oversees reporting of status, problems, and progress to TSSWCB PM.

Kevin Wagner, TWRI Quality Assurance Officer

Responsible for coordinating development and implementation of the TWRI's QA program including writing, maintaining and distributing QAPP and any appendices and amendments, and monitoring its implementation. Ensures data collected for the project is of known and acceptable quality and adheres to the specifications of the QAPP. Responsible for identifying, receiving, and maintaining project quality assurance records. Responsible for coordinating with the TSSWCB to resolve QA-related issues. Notifies the AgriLife Research Project Lead, TWRI Project Co-Lead and TSSWCB PM of particular circumstances which may adversely affect the quality of data. Coordinates the research and review of technical QA material and data related to water quality monitoring system design and analytical techniques. Implements or ensures implementation of corrective actions needed to resolve nonconformance noted during assessments. Provides copies of QAPP and any amendments or revisions to each project participant.

Responsible for the acquisition, verification, and transfer of data to the TSSWCB PM. Oversees data management for the project. Performs data quality assurances prior to transfer of data to TSSWCB. Provides the point of contact for the TSSWCB PM to resolve issues related to the data and assumes responsibility for the correction of any data errors.

Lucas Gregory, TWRI Project Manager

Responsible for project oversight, ensuring that contract requirements are executed on time and are of acceptable quality. Oversees fiscal operations of the project and ensures that expenditures are reasonable and accurate. In charge of project reporting.

Texas AgriLife Research – Soil and Crop Sciences Department

Terry Gentry, Project Lead and SAML Laboratory Director

The Project Lead is responsible for ensuring that tasks and other requirements in the contract are executed on time and with the QA/QC requirements in the system as defined by the contract and in the QAPP; assessing the quality of subcontractor/participant work; and submitting accurate and timely deliverables to the TSSWCB PM. Responsible for ensuring adequate training and supervision of all activities involved in generating analytical and field data. Responsible for supervision of laboratory personnel involved in generating analytical data for the project. Responsible for ensuring that laboratory personnel involved in generating analytical data have adequate training and thorough knowledge of the QAPP and all SOPs specific to the analyses or task performed. Responsible for oversight of all laboratory operations ensuring that all QA/QC requirements are met, documentation related to the analysis is complete and adequately maintained, and that results are reported accurately. Responsible for ensuring that corrective actions are implemented, documented, reported and verified. Monitors implementation of the measures within the laboratory to ensure complete compliance with project DQOs in the QAPP. Conducts in-house audits to ensure compliance with written SOPs and identify potential problems.

Brazos River Authority

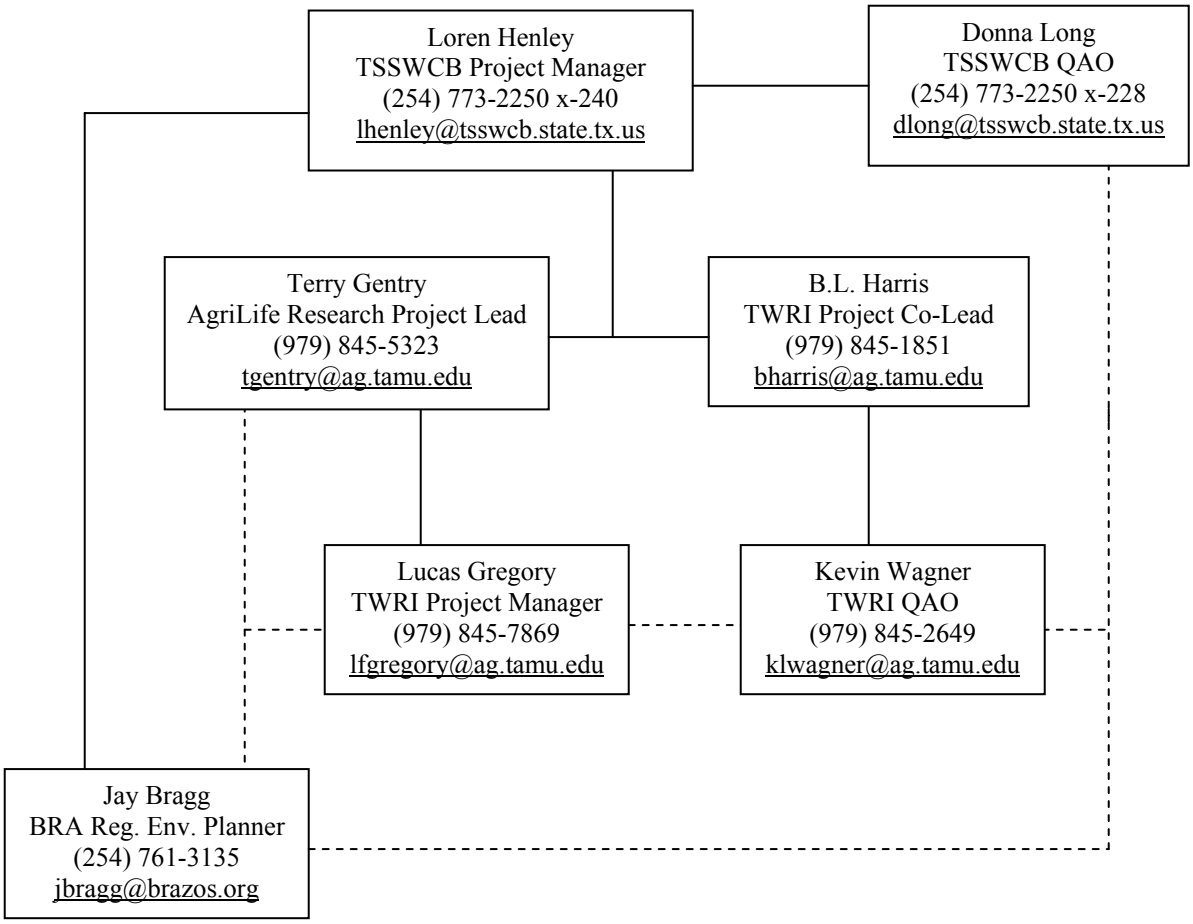
Jay Bragg, Central/Lower Region Environmental Planner

Responsible for overall coordination and cooperation between contractors and sub-contractors on TSSWCB projects *Assessment of Contact Recreation Use Impairments and Watershed Planning for Five Tributaries of the Little Brazos River* (08-54), *Modeling Support for Little Brazos River Tributaries Bacteria Assessment* (08-55) and *Bacterial Source Tracking for Little Brazos River Tributaries Bacteria Assessment* (09-52). Responsible for the timely collection and delivery of QA water quality data, water samples, fecal samples and watershed inventory data to contractors and sub-contractors on TSSWCB projects (08-55) and (09-52). Monitors and assesses the quality of work. Coordinates attendance at conference calls, training, meetings, and related project activities with the TSSWCB.

Tiffany Morgan, Environmental Services Manager

Reports to the BRA Technical Services Manager and oversees field data collections, environmental laboratory operations, and data management activities. The majority of these activities are directly related to a regional project. The Environmental Services Manager provides technical guidance and assistance to regional environmental projects, including initiating, planning, facilitating, and executing of projects. The Environmental Services Manager is solely responsible for the implementation of quality management (planning, assurance, and control) for field, laboratory, and data management operations.

Figure A4.1 Organization Chart



A5 PROBLEM DEFINITION/BACKGROUND

The central watershed of the Brazos River consists of one classified waterbody, the Brazos River above Navasota River (Segment 1242), and a number of unclassified tributaries. This segment extends from the Lake Brazos Dam in Waco 183 miles downstream to its confluence with the Navasota River southeast of College Station. Its watershed encompasses approximately 2,705 mi^2. With the exception of the Waco and Bryan/College Station urban areas, land use in the watershed is generally agricultural with a few large industrial facilities and quarries.

In 2002, water quality data analysis determined that 7 unclassified waterbodies within the central watershed had bacteria concentrations that exceed state water quality standards for contact recreation. These waterbodies were placed on the *Texas 303(d) List*. Four additional unclassified segments were added to the *2006 303(d) List* bringing the total number of water quality impairments (bacteria) on segment 1242 to eleven. All 11 remain on the *2008 303(d) List*.

Of those waterbodies impaired for bacteria, five are located within a very close proximity of each other in Robertson County and share similar land use and water quality characteristics. In addition, they are all tributaries to the Little Brazos River (Segment 1242E). The five waterbodies in this project's study area are Campbells Creek (Segment 1242I), Mud Creek (Segment 1242K), Pin Oak Creek (Segment 1242L), Spring Creek (Segment 1242M), and Walnut Creek (Segment 1242O). The study area encompasses 327 mi^2, almost entirely within Robertson County. The land use in the area is primarily agricultural (range and pastureland with mixed areas of cultivated cropland) with several small communities.

The *2008 303(d) List* identifies three segments in the study area as Category 5a and two segments in the study area as Category 5c, meaning that the waterbody does not meet applicable water quality standards for one or more designated uses by one or more pollutants and that either (5a) a TMDL is underway, scheduled, or will be scheduled, or (5c) additional data and information will be collected before a TMDL is scheduled.

The TCEQ and the TSSWCB established a joint, technical Task Force on Bacteria TMDLs in September 2006 charged with making recommendations on cost-effective and time-efficient bacteria TMDL development methodologies. The Task Force recommended the use of a three-tier approach that is designed to be scientifically credible and accountable to watershed stakeholders. The tiers move through increasingly aggressive levels of data collection and analysis in order to achieve stakeholder consensus on needed load reductions and strategies to achieve those reductions. In June 2007, the TCEQ and the TSSWCB adopted the principles and general process recommended by the Task Force and directed agency staff to incorporate the principles of the recommendations into an updated joint-agency TMDL guidance document.

In accordance with the *Memorandum of Agreement Between the TCEQ and the TSSWCB Regarding TMDLs, Implementation Plans, and Watershed Protection Plans*, the TSSWCB has agreed to take the lead role in addressing the bacteria impairments for the five segments in the study area. Through this and associated projects, the TSSWCB and SCSC will work with local stakeholders to progress through the data collection and analysis components of the first two tiers of the Task Force recommended three-tier approach.

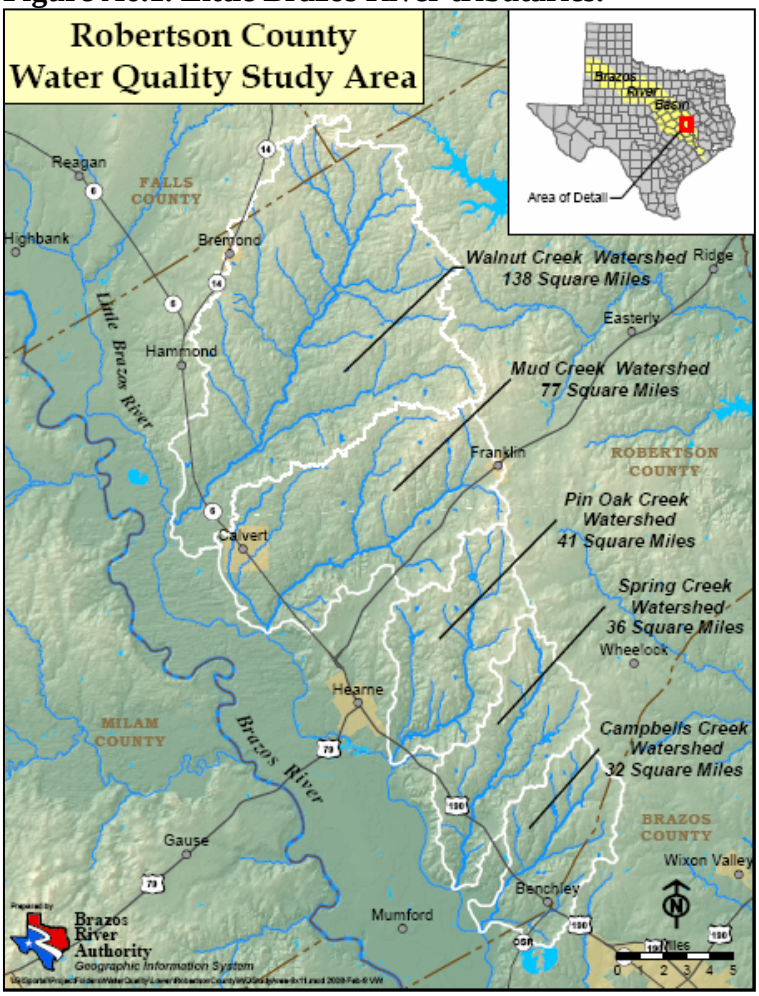
A6 PROJECT/TASK DESCRIPTION

General Project Description

In order to communicate project goals, activities, results and accomplishments to affected parties, SCSC and TWRI will participate in public stakeholder meetings as needed. At a minimum, public stakeholder meetings shall consist of an organizational/kick-off meeting, a source survey design meeting, a meeting presenting results from initial data analysis, a Texas Watershed Steward Program workshop, two project update meetings during the middle of the project, a meeting presenting data analysis results, and a meeting presenting final technical reports.

TWRI will develop a QAPP to ensure data of known and acceptable quality are generated and used in this project. The QAPP shall be consistent with the *TSSWCB Environmental Data Quality Management Plan*.

Figure A6.1. Little Brazos River tributaries.



To assess and identify different sources contributing to bacteria loadings to Little Brazos River tributaries (Figure A6.1), SCSC will conduct BST. SCSC will conduct library-independent BST utilizing the *Bacteroidales* PCR genetic test for human, ruminant, horse, and swine markers. Additionally, SCSC will conduct limited library-dependent BST and analyze *E. coli* isolates utilizing the ERIC-PCR and RiboPrinting (RP) combination method (ERIC-RP). This will serve to confirm that the sources of *E. coli* and *Bacteroidales* are comparable and assess the spatial and temporal adequacy of the Texas Known Source Library in order to determine the need for collection of local known source library samples if intensive library-dependent BST is employed in the future.

To provide sufficient water quality data to characterize bacteria loadings in the study area, BRA, through TSSWCB project 08-54, *Assessment of Contact Recreation Use Impairments and Watershed Planning for Five Tributaries of the Little Brazos River*, will conduct routine ambient

monitoring, effluent monitoring, and biased-flow monitoring under high flow conditions. BRA will provide SCSC a subset of these samples for BST. BRA will work with SCSC to ensure sample collection activities employ adequate QA/QC mechanisms for BST.

BRA will design and conduct a watershed source survey, through TSSWCB project 08-54, that better characterizes the possible sources of bacteria loadings in the study area. SCSC will assist BRA in designing the watershed source survey. Results from the source survey will be used by SCSC to make appropriate adjustments to the BST sampling design and to assess the adequacy of the Texas Known Source Library.

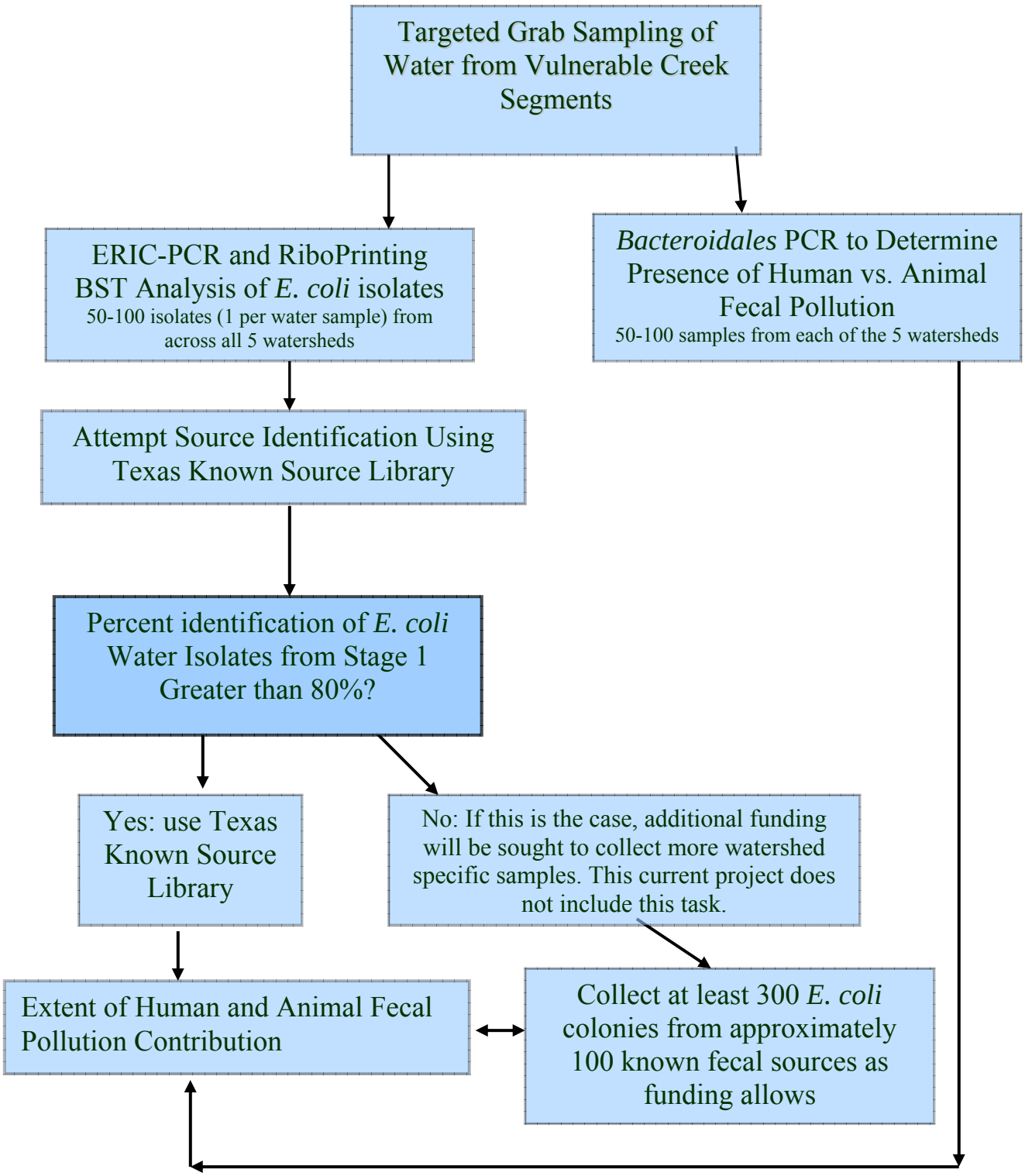
To estimate loadings from various sources and to identify critical loading areas within the watersheds, BAEN will conduct watershed modeling for the study area through TSSWCB project 08-55, *Modeling Support for Little Brazos River Tributaries Bacteria Assessment*. SCSC will work with BAEN to integrate BST results into the model, to the extent possible, and address and reconcile discrepancies between BST and modeling results.

Identification of Sources

New data, of known and specified quality, will be collected and analyzed to differentiate and quantify the relative contributions of livestock, wildlife, and other human and animal *E. coli* sources to Campbells, Mud, Pin Oak, Spring, and Walnut Creeks. This assessment and differentiation between bacteria sources will utilize the BST Texas Known Source Library coordinated by AgriLife El Paso. The library contains diverse *E. coli* isolates that were selected after screening over 4,400 isolates by genetic fingerprinting to exclude identical isolates from the same sample and include isolates with unique genetic fingerprints. This project will provide sufficient documentation of the data and technical analyses conducted that will aid the project staff in communicating the assessment results to watershed stakeholders and TSSWCB.

Fifty to one-hundred *E. coli* isolates from 50-100 different water samples (1 isolate per water sample) collected from across the study area will be analyzed by SAML using the (ERIC-PCR) and RiboPrinting BST methods described below and compared with isolates from the previously developed Texas Known Source Library. Additionally, 50-100 water samples collected from each of the five segments will be analyzed by SAML for *Bacteroidales* PCR markers (general, human, ruminant, swine, and equine). An experimental approach flow diagram is presented in Figure A6.2.

Figure A6.2. Flow Diagram of Experimental Approach for BST



BRA will be responsible for collecting water samples through TSSWCB project 08-54 and delivering a subset of those samples to SAML. SAML will be responsible for processing and isolating *E. coli* from water samples and for pre-processing of water samples for *Bacteroidales* PCR. *E. coli* will be isolated from the samples using standard microbiological methods as previously used in TSSWCB and TCEQ BST projects. *E. coli* will be quantified and then isolated from water samples using EPA Method 1603 and modified membrane Thermotolerant *E. coli* (mTEC) medium. The modified mTEC method is a single-step method that uses one medium and does not require testing using any other substrate.

Limited Library Dependant BST

Enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR), a type of rep-PCR, has moderately high ability to resolve different closely related bacterial strains (Versalovic, Schneider et al. 1994). Consumable costs for ERIC-PCR are inexpensive and labor costs for sample processing and data analyses are moderate. ERIC-PCR is a genetic fingerprinting method used in previous BST studies as well as many microbial ecology and epidemiological studies. ERIC elements are repeat DNA sequences found in varying numbers and locations in the genomes of different bacteria such as *E. coli*. The PCR is used to amplify the DNA regions between adjacent ERIC elements. This generates a DNA banding pattern or fingerprint which looks similar to a barcode pattern. Different strains of *E. coli* bacteria have different numbers and locations of ERIC elements in their bacterial genomes, and therefore, have different ERIC-PCR fingerprints. ERIC-PCR is useful as a screening technique for library development because of its moderate cost and moderately high ability to resolve different strains of the same species of bacteria. Though rep-PCR banding patterns for isolates tend to be generally stable, differences in fingerprint image processing and PCR protocols between laboratories may result in reduced between-laboratory reproducibility and pose a challenge to generating a composite library in multiple laboratories. Rigorous QC and QA, standardized protocols for PCR and image processing, and adequate training of personnel is crucial for generation of comparable data (Bacteria TMDL Task Force Final Report; TWRI TR-341).

Ribotyping is a genetic fingerprinting method used in previous BST studies and many microbial ecology and epidemiological studies. In general, an endonuclease enzyme (*Hind* III) selectively cuts *E. coli* DNA wherever it recognizes a specific DNA sequence. The resulting DNA fragments are separated by size and probed for fragments containing particular conserved ribosomal RNA gene sequences, which results in DNA banding patterns or fingerprints that look similar to barcodes. Different strains of *E. coli* bacteria have differences in their DNA sequences and different numbers and locations of enzyme cutting sites, and therefore have different ribotyping fingerprints. The DuPont Qualicon RiboPrinter Microbial Characterization System allows automation of the ribotyping ('RiboPrinting').

A total of 50-100 *E. coli* isolates obtained from ambient water samples from across the study area will be characterized using ERIC-PCR and RiboPrinting. DNA patterns of those isolates will be compared to the Texas Known Source Library of *E. coli* isolates from known animal and human sources collected throughout Texas. Water isolates will be identified to cattle, other livestock, avian and non-avian wildlife, domestic sewage, and pet sources (six-way split), as well as a broader three-way split of livestock, domestic sewage and wildlife.

Library Independent BST

PCR genetic testing for *Bacteroides* fecal bacteria will be performed by SAML to determine the source of the fecal pollution. The *Bacteroidales* PCR method is a culture-independent molecular method which targets genetic markers of *Bacteroidales* and *Prevotella* spp. fecal bacteria that are specific to humans, ruminants (including cattle and deer), pigs, and horses [Bernhard, A. E. and K. G. Field (2000). "A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA." Appl Environ Microbiol 66(10): 4571-4574**;** Dick, L. K., A. E. Bernhard, et al. (2005). "Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification." Appl Environ Microbiol 71(6): 3184-3191]. The method has high specificity and moderate sensitivity [Field, K. G., E. C. Chern, et al. (2003). "A comparative study of culture-independent, library-independent genotypic methods of fecal source tracking." J Water Health 1(4): 181-94]. For this method, 100 ml water samples are concentrated by filtration, DNA extracted from the concentrate and purified, and aliquots of the purified DNA analyzed by PCR. For pre-processing of water samples for *Bacteroidales* PCR, SAML will filter the water samples, place the filters in DNA lysis buffer and freeze at -80° C until analysis. At the time of analysis, SAML will extract and purify DNA from the filters. DNA extracted from the water samples will be tested for the general, human, ruminant (including cattle and deer), pig (including feral hogs), and horse fecal markers. Results are typically expressed as presence/absence of the host-specific genetic markers; therefore, this method is not quantitative.

Timeline

In order to produce results in a timely manner, the BST will follow the timeline described in Table A6.1.

Table A6.1. Project Plan Milestones

Task	Project Milestones	Agency	Start	End
1.1	Prepare & submit quarterly reports to TSSWCB & participants	TWRI	09/08	05/10
1.2	Perform accounting functions	TWRI & SCSC	09/08	05/10
1.3	Participate in coordination meetings with TSSWCB & partners	TWRI & SCSC	09/08	05/10
1.4	Participate in stakeholder meetings	TWRI & SCSC	09/08	05/10
2.1	Develop QAPP	TWRI	09/08	04/09
2.2	QAPP Annual Revision #1	TWRI	09/09	12/09
3.1	Conduct library-independent BST on 50-100 water samples per each of the 5 watersheds	SCSC	05/09	04/10
3.2	Conduct library-dependent BST from 50-100 water samples across the study area	SCSC	05/09	04/10
3.4	Assist BRA in designing a watershed source survey	SCSC	09/08	05/10
3.5	Assist BAEN in reconciling BST and modeling results	BAEN		
3.6	SWQM	BRA		

A7 QUALITY OBJECTIVES AND CRITERIA

The project objective is to assess contact recreation use impairments and support watershed planning for five tributaries of the Little Brazos River by conducting BST. The measurement performance specifications to support the project objective are specified in Table A7.1. Laboratory measurement QC requirements and acceptability criteria are provided in Section B5.

Table A7.1. Measurement Performance Specifications

Parameter	Method Type	Method	Method Description	Precision Of Laboratory Duplicates[1]	Bias[1]	Percent Complete[2]
E. coli RiboPrinting	DNA/ image matching	EP AREC SOP	RiboPrinting	90% identical	90% correct	90
E. coli ERIC-PCR	DNA/ image matching	EP AREC SOP	ERIC-PCR	90% identical	90% correct	90
Bacteroidales PCR	PCR presence /absence	EP AREC SOP	*Bacteroidales* PCR	100% agreement	90% correct	90
E. coli isolation	Membrane filter culture on modified mTEC agar	USEPA 1603	Membrane Filter	N/A	N/A	N/A

[1] Bias and laboratory method precision will be determined using isolates from known-source samples in a blind procedure, as discussed in Section B5.

[2] The objective is for 90% of the data to be collected. An additional objective for BST completeness is that sources for 70% of host-specific isolates can be identified.

Precision

The precision of laboratory data is a measure of the reproducibility of a result from repeated analyses. It is strictly defined as a measure of the closeness with which multiple analyses of a given sample agree with each other. Precision is assessed by repeated analyses of a sample. Laboratory precision is assessed by comparing sample/duplicate pairs, in the case of bacterial analysis. Precision results are compared against measurement performance specifications and used during evaluation of analytical performance. Measurement performance specifications for precision are defined in Table A7.1.

The ERIC-PCR and RP BST techniques are qualitative assays, generating two different types of DNA fingerprints. For *Bacteroidales* there is a presence/absence qualitative PCR assay. Precision for ERIC-PCR and RP will be determined using a control strain of *E. coli* (QC101), while fecal DNA from known-source samples will be used for *Bacteroidales* PCR. For ERIC-PCR and RP, the DQO is 90% precision. For *Bacteroidales* PCR the DQO is 100% agreement in marker detection among replicates.

Bias

Bias is a statistical measurement of correctness and includes components of systemic error. A measurement is unbiased when the value reported does not differ from the true value. Performance specifications for bias are specified in Table A7.1. In BST, it is best quantified through RP and ERIC-PCR of *E. coli* and PCR of *Bacteroidales* isolated from known sources as

"double-blind" samples selected by a third party. Results are compared against measurement performance specifications and used during evaluation of analytical performance. For the *E. coli* methods the DQO is 90% accuracy for correct identification to library strain or source. For *Bacteroidales* PCR, the DQO is 90% accuracy for the presence/absence of the appropriate markers in known source fecal DNA samples.

An additional element of bias is the absence of contamination. This is determined through the analysis of blank samples of sterile water processed in a manner identical to the sample. Requirements for blank samples are discussed in Section B5.

Sensitivity

Sensitivity is a measure that is used to determine a method's detection limits. The detection limit of quantitative methods is defined as the minimum concentration of a substance that can be measured with a given level of confidence that the analyte concentration is greater than zero (*QA/QC Guidance for Laboratories Performing PCR Analyses on Environmental Samples*. EPA, 2004). For presence/absence methods, the detection limit is the minimum concentration of analyte that produces a positive response with a given level of confidence. The detection limits can be expressed as the minimum number of organisms or of the target sequence copy number in a given volume. Many uncertainties can affect the detection limit; some are the:

- type of target nucleic acid being detected (e.g. DNA, mRNA, tRNA, etc.),
- secondary structure and the GC content of the nucleic acid target molecule,
- matrix from which the organism is located, and
- detection of microbes that are inactivated by physical and chemical disinfectants.

For analyzing environmental samples using PCR, the detection limits can be for the limit of the entire method or the limit of the PCR procedure.

Representativeness

Data collected under this project will be considered representative of ambient water quality conditions. Representativeness is a measure of how accurately a monitoring program reflects the actual water quality conditions typical of receiving water. The representativeness of the data is dependent on 1) the sampling locations, 2) the number of samples collected, 3) the number of years and seasons when sampling is performed, 4) the number of depths sampled, and 5) the sampling procedures. Site selection procedures will assure that the measurement data represent the conditions at the site. The goal for meeting total representation of the waterbody and watershed is tempered by the availability of time, site accessibility, and funding. Representativeness will be measured with the completion of sample collection in accordance with the approved QAPP. Sample collection is dictated by TSSWCB QAPP 08-54.

Comparability

The comparability of the data produced is predetermined by the commitment of the staff to use only approved procedures as described in this QAPP. Comparability is also guaranteed by reporting all ambient, high flow, and QC data for evaluation by others.

Completeness

The completeness of the data is a measure of how much of the data is available for use compared with the total potential data. Ideally, 100% of the data would be available. However, the possibility of unavailable data due to accidents, weather, insufficient sample volume, broken or lost samples, etc. is to be expected. Therefore, it will be a general goal of the project(s) that 90% data completion is achieved.

Should less than 90% data completeness occur, the TWRI PM will initiate corrective action. Data completeness will be calculated as a percent value and evaluated with the following formula:

% completeness = (SV X 100) / ST

Where: SV = number of samples with a valid analytical report
ST = total number of samples collected

An additional element of completeness is involved with BST. The sources of *E. coli* isolates which do not match those from a library of known sources cannot be identified. In all BST studies, a source cannot be identified with acceptable confidence for a portion of the *E. coli* isolates. This is a function of 1) the size of the library relative to the true diversity of *E. coli* in the watershed, 2) the ability of the method to distinguish sources with acceptable confidence, and 3) the abundance of *E. coli* strains that colonize multiple sources, and thus cannot be used to uniquely identify a source. It will be a general goal of this project to identify the sources of 80% of the *E. coli* strains isolated from water and 90% of the *Bacteroidales* for the general marker in all samples that have a countable number of *E. coli*.

A8 SPECIAL TRAINING/CERTIFICATION

All personnel involved in sample analyses and statistical analyses have received the appropriate education and training required to adequately perform their duties. No special certifications are required. SAML personnel involved in this project have been trained in the appropriate use of laboratory equipment, laboratory safety, cryogenics safety, and all applicable SOPs. Each laboratory analyst must demonstrate their capability to conduct each test that the analyst performs to the Lab Director. This demonstration of capability is performed before analyzing samples and annually thereafter. Finally, SAML is in the process of becoming NELAC-certified for enumerating *E. coli* in both non-potable and drinking water using USEPA Method 1603.

A9 DOCUMENTS AND RECORDS

The document and records that describe, specify, report, or certify activities, requirements, procedures, or results for this project and the items and materials that furnish objective evidence of the quality of items or activities are listed in Table A9.1.

Table A9.1 Project Documents and Records

Document/Record	Location	Retention	Form
QAPP, amendments, and appendices	TWRI	5 years	Paper/Electronic
COC records	SAML	5 years	Paper
Corrective action reports	TWRI	5 years	Paper
Bacteriological data log sheet	SAML	5 years	Paper
Laboratory QA manuals	SAML	5 years	Paper
Laboratory SOPs	SAML	5 years	Paper
Instrument raw data files, readings and printouts	SAML	5 years	Paper/Electronic
Lab equipment calibration records & maintenance logs	SAML	5 years	Paper
Lab data reports	TWRI/TSSWCB	3 years	Paper/Electronic
Progress reports/final report/data	TWRI/TSSWCB	3 years	Paper/Electronic

Individual laboratory notebooks, which contain printouts of laboratory data and hand written observations and data, are kept by individual analysts at SAML or the AgriLife Research Project Lead for at least five years. When lab notebooks are filled, they are stored for at least five years by the AgriLife Research Project Lead/Laboratory Manager in hardcopy form. The SAML keeps electronic data on personal computers for the duration of the project and then in hardcopy files for 5 years after the project. COCs and attached documents are stored in numerical order in three-ring binders in the AgriLife Research Project Lead/Laboratory Manager's office for at least five years. In addition, the AgriLife Research Project Lead/Laboratory Manager will archive electronic forms of all project data for at least five years on personal computers and fire-resistant cabinets. Lab data reports from the SAML, as included in the final report, and other reports as required, will report test results clearly and accurately.

QPRs will note activities conducted in connection with BST, items or areas identified as potential problems, and any variations or supplements to the QAPP. CARs will be utilized when necessary. CARs that result in any changes or variations from the QAPP will be made known to pertinent project personnel and documented in an update or amendment to the QAPP. All QPRs and QAPP revisions will be distributed to personnel listed in Section A3.

All electronic data are backed up on an external hard drive monthly, compact disks weekly, and is simultaneously saved in an external network folder and the computer's hard drive. A blank CAR is presented in Appendix A and a blank COC form is presented in Appendix C.

The TSSWCB may elect to take possession of records at the conclusion of the specified retention period.

QAPP Revision and Amendments

Until the work described is completed, this QAPP shall be revised as necessary and reissued at least annually on the anniversary date, or revised and reissued within 60 days of significant changes, whichever is sooner. The last approved versions of QAPPs shall remain in effect until revised versions have been fully approved; the revision must be submitted to the TSSWCB for approval before the last approved version has expired. If the entire QAPP is current, valid, and accurately reflects the project goals and the organization's policy, the annual re-issuance may be done by a certification that the QAPP is current. This will be accomplished by editing the QAPP to update the revision number and date and then submitting a cover letter stating the status of the QAPP and a copy of new, signed approval pages for the QAPP.

QAPP amendments may be necessary to reflect changes in project organization, tasks, schedules, objectives and methods; address deficiencies and nonconformances; improve operational efficiency; and/or accommodate unique or unanticipated circumstances. Written requests for amendments that do not constitute a significant change are directed from the TWRI PM/QAO to the TSSWCB PM and are effective immediately upon approval by the TSSWCB PM and QAO. Amendments to the QAPP and the reasons for the changes will be documented and distributed to all individuals on the QAPP distribution list by the TWRI PM/QAO. Amendments shall be reviewed, approved, and incorporated into a revised QAPP during the annual revision process.

B1 SAMPLING PROCESS DESIGN

To provide sufficient water quality data to characterize bacteria loadings across the various flow regimes, BRA, under the *Assessment of Contact Recreation Use Impairments and Watershed Planning for Five Tributaries of the Little Brazos River* project (TSSWCB project 08-54) will conduct routine ambient monitoring once every two weeks at ten sites. When authorization to enter wastewater treatment facilities (WWTF) at Calvert, Bremond and Franklin is obtained from both the TCEQ and the respective municipalities, BRA will conduct effluent monitoring at the three WWTFs once every two weeks in an effort to estimate possible contributions from wastewater discharges. BRA will conduct biased-flow monitoring under high flow (storm event) conditions at the same ten stream sites and three WWTFs during at least twelve storm events. BRA will conduct biological monitoring on the Little Brazos River below the confluence of the tributaries to assess the cumulative impact of the impaired segments on stream health and biological communities.

Field data and samples will be collected following procedures detailed in the *TCEQ SWQM Procedures, Volume 1 (RG-415).*

BRA will provide a subset of collected water samples to SAML for BST (Table B1.1). SAML will perform *Bacteroidales* PCR on approximately 244 individual water samples collected by BRA between May 2009 and April 2010. The samples will include: 1) 18 sample events for each of the 10 stream sites; 2) 4 sample events for each of the 3 WWTFs; and 3) 4 sample events for each of the 10 stream sites and 3 WWTFs during storm events. SAML will also isolate and fingerprint (ERIC-RP) *E. coli* (one per site per sample event) from each of the 10 stream sites and 3 WWTFs for 4 sample events and also 2 storm events.

Table B1.1. Samples to be Analyzed using *Bacteroidales* PCR and ERIC-RP.

	2009								**2010**			
Parameter	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
Bacteroidales												
Stream (10)[1]	X	XX	X	XX	X	XX	XX	X	X	XX	XX	X
WWTFs (3)		X			X			X			X	
Storm - Stream (10)[2]		X			X			X			X	
Storm - WWTFs (3)		X			X			X			X	
E. coli (ERIC-RP)												
Stream (10)		X			X			X			X	
WWTFs (3)		X			X			X			X	
Storm - Stream (10)[2]		X						X				
Storm - WWTFs (3)		X						X				

[1] For months were two sets of stream samples will be analyzed, the second set of samples will have been collected at least two weeks after the first set for that month and at least two weeks before the next month's samples.

[2] Approximately one storm event sample will be analyzed per site per quarter using *Bacteroidales* PCR and every other quarter using ERIC-RP.

B2 SAMPLING METHODS

BRA will conduct water quality monitoring in the study area through TSSWCB project 08-54. Samples will only be taken if water is flowing. If a site is not flowing but pooled or dry, that will be noted on the field data sheet. Water samples will be collected directly from the stream (midway in the stream channel) into containers as specified in Table B2.1. The sample container will be held upstream of the sampler and care will be taken to avoid contact with sediment and the surface micro layer of water. All samples will be transported in an iced container to SAML for analysis.

Table B2.1. Container, Preservation, Temp., Sample Size, and Holding Time Requirements

Parameter	Matrix	Container	Preservation	Temp.	Sample Size	Holding Time
E. coli	water	IDEXX bottle	none	4°C	100 ml	8 hours[1]
E. coli water isolates	NA- MUG agar	Petri dish 100mm x 15mm	20% glycerol; 80% tryptic soy broth	44.5°C	5 colony streaks	20 – 24 hrs, then frozen indefinitely
Bacteroidales	Supor filters	15 ml centrifuge tube	GITC buffer	4°C	100 ml	6 hours[1], filters indefinitely

[1] 8 hours to deliver to laboratory. In the case that this 8-hour holding time is not met, the *E. coli* quantitative count will be flagged and not reported, though the *Bacteroidales* PCR will still be valid.

Recording Data

For the purposes of this section and subsequent sections, all field and laboratory personnel will: (1) write legibly in indelible, waterproof ink with no modifications, write-overs or cross-outs; (2) correct errors with a single line followed by an initial and date; and (3) close-out incomplete pages with an initialed and dated diagonal line.

Deviations from Sampling Method Requirements or Sample Design, and Corrective Action

Examples of deviations from sampling method requirements include inadequate sample volume collected, failure to preserve samples appropriately, contamination of sample bottle during collection, storage temperature and holding time exceedance, and sampling at the wrong site. Deviations invalidate resulting data and may require corrective action including samples being discarded and re-collected. It is the responsibility of the SAML Project Leader and TWRI QAO to ensure that the actions and resolutions to the problems are documented and that records are maintained in accordance with this QAPP. In addition, these actions and resolutions will be conveyed to the TSSWCB PM both verbally and in writing in QPRs and by completion of a CAR as shown in Appendix A. CARs will be included with QPRs. In addition, significant conditions (i.e., situations which, if uncorrected, could have a serious effect on safety or on the validity or integrity of data) will be reported to the TSSWCB immediately both verbally and in writing.

B3 SAMPLE HANDLING AND CUSTODY

Chain of Custody

Proper sample handling and custody procedures ensure the custody and integrity of samples beginning at the time of sampling and continuing through transport, sample receipt, preparation, and analysis. The COC form is used to document sample handling during transfer from the field to the laboratory. The sample number, location, date, changes in possession and other pertinent data will be recorded in indelible ink on the COC. The sample collector (BRA) will sign the COC and transport it with the sample to the laboratory (SAML). At the laboratory, samples are inventoried against the accompanying COC. Any discrepancies will be noted at that time and the COC will be signed for acceptance of custody. A blank COC form used on this project is included as Appendix C.

Sample Labeling

Samples will be labeled on the container with an indelible, waterproof marker. Label information will include site identification, date, sampler's initials, and time of sampling. The COC form will accompany all sets of sample containers.

Sample Handling

Following collection, samples will be placed on ice in an insulated cooler for transport to the laboratory. At the laboratory, samples will be placed in a refrigerated cooler dedicated to sample storage. The Laboratory Director has the responsibility to ensure that holding times are met with water samples. The holding time is documented on the COC. Any problem will be documented with a CAR.

Failures in Chain of Custody and Corrective Action

All failures associated with COC procedures as described in this QAPP are immediately reported to the TWRI PM/QAO. These include such items as delays in transfer, resulting in holding time violations; violations of sample preservation requirements; incomplete documentation, including signatures; possible tampering of samples; broken or spilled samples, etc. The TWRI PM/QAO will determine if the procedural violation may have compromised the validity of the resulting data. Any failures that have reasonable potential to compromise data validity will invalidate data and the sampling event should be repeated. The resolution of the situation will be reported to the TSSWCB PM in the QPR. CARs will be prepared by the TWRI PM/QAO and submitted to the TSSWCB PM along with QPRs.

B4 ANALYTICAL METHODS

The analytical methods are listed in Table B4.1 and described in detail in Appendix B.

E. coli in water samples will be quantified and isolated by SAML personnel using modified mTEC agar, EPA Method 1603 [EPA/821/R-02/023. September 2002. *Escherichia coli* in Water by Membrane Filtration Using Modified Membrane-Thermotolerant *Escherichia coli* (modified m-TEC) Agar]. The modified medium contains the chromogen 5-bromo-6-chloro-3-indolyl-β-D-glucuronide (Magenta Gluc), which is catabolized to glucuronic acid (a red/magenta-colored compound) by *E. coli* that produces the enzyme β-D-glucuronidase. This enzyme is the same enzyme tested for using other substrates such as the fluorogenic reaction with 4-methylumbelliferyl-β-D-glucuronide (MUG) observed by ultraviolet light (UV) fluorescence. *E. coli* colonies from the modified mTEC medium will be picked and streaked for purity on nutrient agar with MUG (NA-MUG) to confirm glucuronidase activity and culture purity. Cultures of selected isolates will be archived using glycerol freezing medium (-80^{o}C). Inoculated plates will be incubated at 35±0.5^{o}C for 2 hours to resuscitate stressed bacteria, then incubated at 44.5±0.2^{o}C for approximately 20 to 24 hours. *E. coli* isolates obtained from ambient water samples from across the study area will be characterized using ERIC-PCR and RiboPrinting using AgriLife El Paso SOPs. DNA patterns of those isolates will be compared to the Texas Known Source Library of *E. coli* isolates from known animal and human sources collected throughout Texas. Water isolates will be identified to cattle, other livestock, avian and non-avian wildlife, domestic sewage, and pet sources (six-way split), as well as a broader three-way split of livestock, domestic sewage and wildlife.

Table B4.1. Laboratory Analytical Methods

Parameter	Method	Equipment Used
E. coli	EPA 1603	Filtration apparatus, incubator
E. coli RiboPrint fingerprint	AgriLife El Paso SOP	RiboPrinter
E. coli ERIC-PCR fingerprint	AgriLife El Paso SOP	PCR thermal cycler, gel electrophoresis apparatus
Bacteroidales PCR	AgriLife El Paso SOP	PCR thermal cycler, gel electrophoresis apparatus

EPA = Methods for Chemical Analysis of Water and Wastes, March 1983
SOP = Standard Operating Procedure

As outlined in Appendix B, 100 ml water samples will be collected and filtered for analysis of *Bacteroidales. Bacteriodales* DNA will be extracted from the filters and analyzed using PCR using El Paso AgriLife Research and Extension Center (EP AREC) SOPs.

All laboratory sampling areas and equipment will be sterilized with at least one or in any combination of the following methods: ethyl alcohol, bleach, UV light, or autoclave. All disposables will be placed in a heat-resistant biohazard bag and autoclaved prior to disposal.

Failures in Measurement Systems and Corrective Actions

Failures in field and laboratory measurement systems involve, but are not limited to such things as instrument malfunctions, failures in calibration, blank contamination, QC samples outside QAPP defined limits, etc. In many cases, the field technician or lab analyst will be able to correct the problem. If the problem is resolvable by the field technician or lab analyst, then they will

document the problem on the field data sheet or laboratory record and complete the analysis. If the problem is not resolvable, then it is conveyed to the SAML Director, who will make the determination in coordination with the TWRI QAO. If the analytical system failure may compromise the sample results, the resulting data will not be reported to the TSSWCB as part of this project. The nature and disposition of the problem is reported on the data report. The TWRI PM/QAO will include this information in the CAR and submit with the QPR which is sent to the TSSWCB PM.

B5 QUALITY CONTROL

Table A7.1 lists the required accuracy, precision, and completeness limits for the parameters of interest. It is the responsibility of the SCSC Project Leader to verify that the data are representative. The SCSC Project Leader also has the responsibility of determining that the 90 percent completeness criteria is met, or will justify acceptance of a lesser percentage. All incidents requiring corrective action will be documented through use of CARs (Appendix A). Laboratory audits, sampling site audits, and QA of field sampling methods will be conducted by the TSSWCB QAO or their designee.

Laboratory Blanks

Laboratory blanks, or negative controls, consist of 100-ml aliquots of sterile distilled water that are processed in the same manner as a field sample, at the beginning and the end of a sample set. They are used to assess the sterilization techniques employed throughout the sample process. Laboratory blanks will be included at the beginning and the end of the sample set for each sampling event. The analysis of laboratory blanks should yield a value of no colonies detected. For *Bacteroidales* PCR, a laboratory blank will be analyzed with each batch of samples to ensure no cross-contamination occurs during sample processing. In addition, negative controls will be analyzed for each batch of PCR samples.

Positive Control

Positive controls will be analyzed by SAML for each batch of *E. coli* ERIC-PCR and RiboPrinting, and *Bacteroidales* PCR samples. SAML will maintain live *E. coli* in tryptic soy broth and kept refrigerated until needed. Each time a set of samples is run a positive control will be performed in the lab using the same media and 1 ml of live *E. coli* which will be added to 99 ml of sterile distilled water that will be run through the filter funnel system and the filter placed on the media. This control should always be positive for *E. coli* after recommended incubation time. In addition, positive controls will be analyzed for each batch of *E. coli* ERIC-PCR and RiboPrinting, and *Bacteroidales* PCR.

Laboratory Duplicate

Laboratory duplicates are used to assess precision. A laboratory duplicate is prepared by splitting aliquots of a single sample (or a matrix spike or a laboratory control standard) in the laboratory. Both samples are carried through the entire preparation and analytical process. Laboratory duplicates are run at a rate of one per batch. Acceptability criteria are outlined in Table A7.1 of Section A7.

Precision is calculated by the relative percent difference (RPD) of duplicate results as defined by 100 times the difference (range) of each duplicate set, divided by the average value (mean) of the set. For duplicate results, X_1 and X_2, the RPD is calculated from the following equation:

$$\mathbf{RPD} = \frac{(X_1 - X_2) \times 100}{(X_1 + X_2) \div 2}$$

A bacteriological duplicate is considered to be a special type of laboratory duplicate and applies when bacteriological samples are run in the field as well as in the laboratory. Bacteriological duplicate analyses are performed on samples from the sample bottle on a 10% basis. Results of bacteriological duplicates are evaluated by calculating the logarithm of each result and determining the range of each pair.

Performance limits and control charts are used to determine the acceptability of duplicate analyses. Precision limits for bacteriological analyses are defined in Table A7.1 and applies to samples with concentrations >10 cfu/100 ml.

Failures in Quality Control and Corrective Action

Notations of blank contamination will be noted in QPRs and the final report. Corrective action will involve identification of the possible cause (where possible) of the contamination failure. Any failure that has potential to compromise data validity will invalidate data, and the sampling event should be repeated. The resolution of the situation will be reported to the TSSWCB in the QPR. The CARs will be maintained by the SCSC Project Leader and the TSSWCB PM.

B6 INSTRUMENT/EQUIPMENT TESTING, INSPECTION AND MAINTENANCE

To minimize downtime of all measurement systems, spare parts for laboratory equipment will be kept in the laboratory, and all laboratory equipment must be maintained in a working condition. All laboratory equipment will be tested, maintained, and inspected in accordance with manufacturer's instructions and recommendation in *Standard Methods for the Examination of Water and Wastewater, 21st Edition*. Maintenance and inspection logs will be kept on each piece of laboratory equipment.

All field equipment used to collect samples for this BST will be tested, inspected and maintained in accordance with the TSSWCB QAPP 08-54.

Records of all tests, inspections, and maintenance will be maintained and log sheets kept showing time, date, and analyst signature. These records will be available for inspection by the TSSWCB.

Failures in any testing, inspections, or calibration of equipment will result in a CAR and resolution of the situation will be reported to the TSSWCB in the QPR. The CARs will be maintained by the SCSC Project Leader and the TSSWCB PM.

Table B6.1. Equipment Inspection and Maintenance Requirements

Equipment	Relevant Testing, Inspection & Maintenance Requirement
Thermometers	SM 9020 B 3.a
PCR Thermal cycler	Per manufacturer & annual preventative maintenance
RiboPrinter	Per manufacturer & annual preventative maintenance
Water deionization units	SM 9020 B 3.d
Media dispensing apparatus	SM 9020 B 3.f
Autoclaves	SM 9020 B 3.h
Refrigerator	SM 9020 B 3.i
Ultra Low Freezer	SM 9020 B 3.j
Membrane filter equipment	SM 9020 B 3.k
Ultraviolet sterilization lamps	SM 9020 B 3.l
Biological safety cabinet	SM 9020 B 3.m
Incubators	SM 9020 B 3.o
Glassware and plastic ware	SM 9020 B 4.a
Utensils and containers	SM 9020 B 4.b
Dilution water bottles	SM 9020 B 4.c

B7 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

All instruments or devices used in obtaining environmental data will be calibrated prior to use. Each instrument has a specialized procedure for calibration and a specific type of standard used to verify calibration. The instruments requiring calibration are listed below in Table B7.1.

All calibration procedures will meet the requirements specified in the approved methods of analysis. The frequency of calibration as well as specific instructions applicable to the analytical methods recommended by the equipment manufacturer will be followed. All information concerning calibration will be recorded in a calibration logbook by the person performing the calibration and will be accessible for verification during a laboratory audit.

All instruments or devices used in obtaining environmental data will be used according to appropriate laboratory practices. Written copies of SOPs are available for review upon request.

Standards used for instrument or method calibrations shall be of known purity and be NIST traceable whenever possible. When NIST traceability is not available, standards shall be of ACS or reagent grade quality, or of the best attainable grade. All certified standards will be maintained traceable with certificates on file in the laboratory. Dilutions from all standards will be recorded in the standards log book and given unique identification numbers. The date, analyst initials, stock sources with lot number and manufacturer, and how dilutions were prepared will also be recorded in the standards log book.

Failures in any testing, inspections, or calibration of equipment will result in a CAR and resolution of the situation will be reported to the TSSWCB in the QPR. The CARs will be maintained by the SCSC Project Leader and the TSSWCB PM.

Table B7.1. Instrument Calibration Requirements

Equipment	Relevant Calibration Requirement
RiboPrinter	Per manufacturer & annual preventative maintenance
PCR Thermal Cycler	Per manufacturer & annual preventative maintenance

B8 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

All standards, reagents, media, plates, filters, and other consumable supplies are purchased from manufacturers with performance guarantees, and are inspected upon receipt for damage, missing parts, expiration date, and storage and handling requirements. Labels on reagents, chemicals, and standards are examined to ensure they are of appropriate quality, initialed by staff member and marked with receipt date. Volumetric glassware is inspected to ensure class "A" classification, where required. Media will be checked as described in QC procedures. All supplies will be stored as per manufacturer labeling and discarded past expiration date. In general, supplies for microbiological analysis are received pre-sterilized, used as received, and not re-used.

B9 NON-DIRECT MEASUREMENTS

Historical data will be retrieved from the TCEQ SWQM Information System (SWQMIS). Historical data were collected and analyzed consistently with TCEQ *SWQM Procedures* under the SWQM QAPP or Clean Rivers Program QAPP or EPA approved Brazos/Navasota QAPP and therefore are considered representative of ambient conditions and will be comparable to data collected under this project. Table B9.1 shows the date range of data for each of six existing sites for which SWQMIS has historical data. The mean and median will be computed for each parameter as well as the number of water quality criteria exceedances, as applicable. This information will be compared statistically to the results of data collected under this project. Due to the historical data's comparability to the data collected under this project, there are not limitations on their use.

Table B9.1 Historical Data

Site Number	Site Name	Date Range of Historical Data
11591	Little Brazos River @ SH 21	05/97 – 08/99 and 09/07 – 08/08
16394	Spring Creek @ SH 6/US 190	09/99 – 08/07
16395	Campbells Creek @ Old Hearne Rd	09/99 – 08/00 and 09/05 – 08/08
16401	Pin Oak Creek @ downstream of SH 6	09/98 – 08/07
16402	Mud Creek @ SH 6	09/98 – 08/07
16403	Walnut Creek @ SH 6	09/98 – 08/07

Additionally, data collected by BRA under the *Assessment of Contact Recreation Use Impairments and Watershed Planning for Five Tributaries of the Little Brazos River* project (TSSWCB Project 08-54) will be collected in accordance with that approved QAPP. Data utilized from this project will include water quality samples collected from designated stream crossings and WWTFs and delivered to the SAML within required holding times.

B10 DATA MANAGEMENT

Field Collection and Management of Routine Samples

All field collection to be completed by BRA as outlined in the TSSWCB QAPP 08-54. A field data sheet is filled out in the field for each site visit. Samples collected will be labeled and placed in an iced, insulated chest for transportation to the laboratory. A blank COC form is presented in Appendix C. Site name, time of collection, comments, and other pertinent data are copied from the field data sheet to the COC.

Laboratory Data

Once the samples are received at SAML, samples are logged and stored at 4ºC until processed. The COC will be checked for number of samples, proper and exact ID number, signatures, dates, and type of analysis specified. BRA will be notified if any discrepancy is found and proper corrections made. The COC and accompanying sample bottles are submitted to the SAML laboratory analyst, with relinquishing and receiving personnel both signing and dating the COC. All COC and bacteriological data will be manually entered into an electronic spreadsheet. The electronic spreadsheet will be created in Microsoft© Excel software on an IBM-compatible microcomputer with a Windows® operating system. The project spreadsheet will be maintained on the computer's hard drive, which is also simultaneously saved in a network folder. Data manually entered in the database will be reviewed for accuracy by the SCSC Project Lead or TWRI QAO to ensure that there are no transcription errors. Hard copies of data will be printed and housed in the laboratory for a period of five years. Any COCs and bacteriological records related to QA/QC of bacteriological procedures will be housed at the SAML. All pertinent data files will be backed up monthly on an external hard drive. Current data files will be backed up on an external hard drive monthly and stored in separate area away from the computer. Original data recorded on paper files will be stored for at least five years. Electronic data files will be archived to CD after approximately the end of the project, and then stored with the paper files for the remaining 4 years.

Data Validation

Following review of laboratory data, any data that is not representative of environmental conditions, because it was generated through poor field or laboratory practices, will not be submitted to the TSSWCB. This determination will be made by the SCSC Project Leader, TWRI QAO, TSSWCB QAO, and other personnel having direct experience with the data collection effort. This coordination is essential for the identification of valid data and the proper evaluation of that data. The validation will include the checks specified in Table D2.1.

Data Dissemination

At the conclusion of the project, the SCSC Project Leader will provide a copy of the complete project electronic spreadsheet via recordable CD media to the TSSWCB PM, along with the final report. The TSSWCB may elect to take possession of all project records. However, summaries of the data will be presented in the final project report.

C1 ASSESSMENTS AND RESPONSE ACTIONS

The following table presents types of assessments and response actions for data collection activities applicable to the QAPP.

Table C1.1 Assessments and Response Actions

Assessment Activity	Approximate Schedule	Responsible Party	Scope	Response Requirements
Status Monitoring Oversight	Continuous	TWRI Project Manager	Monitoring of project status and records to ensure requirements are being fulfilled. Monitoring and review of laboratory performance and data quality	Report to TSSWCB in QPR. Ensure project requirements are being fulfilled.
Laboratory Inspections	At least one per life of the project	TSSWCB QAO	Analytical and QC procedures employed at laboratory	30 days to respond in writing to TSSWCB to address corrective actions

Corrective Action

The TWRI QAO and SAML Project Leader are responsible for implementing and tracking corrective action as a result of audit findings. Records of audit findings and corrective actions are maintained by the TSSWCB PM and TWRI QAO. Corrective action documentation will be submitted to the TSSWCB PM with the QPR. If audit findings and corrective actions cannot be resolved, then the authority and responsibility for terminating work is specified in agreements or contracts between participating organizations.

C2 REPORTS TO MANAGEMENT

QPRs will be generated by TWRI and will note activities conducted in connection with BST, items or areas identified as potential problems, and any variation or supplement to the QAPP. CARs will be utilized when necessary (Appendix A) and will be maintained in an accessible location for reference at TWRI. The CARs that result in changes or variations from the QAPP will be made known to pertinent project personnel, documented in an update or amendment to the QAPP and distributed to personnel listed in Section A3. Following any audit performed by TWRI, a report of findings, recommendations and responses are sent to the TSSWCB PM in the QPR.

Field measurements and all sampling for the project will be done according to the QAPP. However, if the procedures and guidelines established in this QAPP are not successful, corrective action is required to ensure that conditions adverse to quality data will be identified promptly and corrected as soon as possible. Corrective actions include identification of root causes of problems and successful correction of identified problems. CARs will be filled out to document the problems and the remedial action taken.

Laboratory data reports contain the results of all analyses, as well as specified QC measures listed in section B5. This information is reviewed by the TWRI QAO and compared to the pre-specified acceptance criteria to determine acceptability of data. This information is available for inspection by the TSSWCB.

The final report for this project will be a technical report detailing the results of BST work conducted under this QAPP. Items in this report will include a very brief description of methodologies utilized, a detailed narrative regarding specific BST findings and a discussion/conclusions section that highlights the implications of the BST findings.

D1 DATA REVIEW, VERIFICATION AND VALIDATION

All data obtained from field and laboratory measurements will be reviewed and verified for conformance to project requirements, and then validated against the DQOs which are listed in Section A7. Only those data which are supported by appropriate QC data and meet the DQOs defined for this project will be considered acceptable. This data will be submitted to the TSSWCB.

The procedures for verification and validation of data are described in Section D2. The SAML Director is responsible for ensuring that laboratory data are scientifically valid, defensible, of acceptable precision and accuracy, and reviewed for integrity. The TWRI PM/QAO will be responsible for ensuring that all data are properly reviewed and verified, validated, and submitted in the required format as described by the TSSWCB PM. Finally, the TWRI PM/QAO is responsible for validating that all data to be reported meet the objectives of the project and are suitable for reporting to TSSWCB.

D2 VERIFICATION AND VALIDATION METHODS

All field and laboratory data will be reviewed, verified and validated to ensure they conform to project specifications and meet the conditions of end use as described in Section A7. The staff and management of the respective field, laboratory, and data management tasks are responsible for the integrity, validation and verification of the data each task generates or handles throughout each process. The field and laboratory tasks ensure the verification of raw data, electronically generated data, and data on COC forms and hard copy output from instruments.

Verification, validation and integrity review of data will be performed using self-assessments and peer review, as appropriate to the project task, followed by technical review by the manager of the task. The data to be verified (listed by task in Table D2.1) are evaluated against project specifications (Section A7) and are checked for errors, especially errors in transcription, calculations, and data input. Potential outliers are identified by examination for unreasonable data. If a question arises or an error or potential outlier is identified, the manager of the task responsible for generating the data is contacted to resolve the issue. Issues which can be corrected are corrected and documented electronically or by initialing and dating the associated paperwork. If an issue cannot be corrected, the task manager consults with the TWRI PM to establish the appropriate course of action, or the data associated with the issue are rejected.

The SCSC Project Lead, with assistance from the TWRI QAO, is responsible for validating that the verified data are scientifically valid, legally defensible, of known precision, accuracy, integrity, meet the DQOs of the project, and are reportable to TSSWCB. One element of the validation process involves evaluating the data for anomalies. The SCSC Project Lead may designate other experienced water quality experts (BRA Environmental Planner or other designated employee) familiar with the waterbodies under investigation to perform this evaluation. Any suspected errors or anomalous data must be addressed by the manager of the task associated with the data, before data validation can be completed.

A second element of the validation process is consideration of any findings identified during the monitoring systems audit conducted by the TWRI QAO or TSSWCB QAO. Any issues requiring corrective action must be addressed, and the potential impact of these issues on previously collected data will be assessed. Finally, the TWRI PM/QAO validates that the data meet the DQOs of the project and are suitable for reporting to the TSSWCB.

Table D2.1. Data Verification Procedures

Data to be Verified	TWRI QAO	SAML Director	TSSWCB PM/QAO
Analysis techniques consistent with SOPs and QAPP	X	X	X
Instrument calibration data complete	X	X	X
Bacteriological records complete		X	X
Sample documentation complete	X	X	X
Sample identifications	X	X	X
COC complete/acceptable	X	X	X
Sample preservation and handling	X	X	X
Holding times	X	X	X
QC samples analyzed at required frequencies		X	X
QC samples within acceptance limits		X	X
Instrument readings/printouts	X	X	X
Calculations	X	X	X
Laboratory data verification for integrity, precision, accuracy, and validation		X	X
Laboratory data reports		X	X
Data entered in required format	X	X	X
Site ID number assigned			X
Absence of transcription error	X	X	X
Reasonableness of data	X	X	X
Electronic submittal errors	X	X	X
Sampling and analytical data gaps	X	X	X

D3 RECONCILIATION WITH USER REQUIREMENTS

Data produced by this project will be evaluated against the established DQOs and user requirements to determine if any reconciliation is needed. Reconciliation concerning the quality, quantity or usability of the data will be reconciled with the user during the data acceptance process. CARs will be initiated in cases where invalid or incorrect data have been detected. Data that have been reviewed, verified, and validated will be summarized for their ability to meet the DQOs of the project and the informational needs of water quality agency decision-makers and watershed stakeholders.

The final data for the project will be reviewed to ensure that it meets the requirements as described in this QAPP. Data summaries along with descriptions of any limitations on data use will be included in the final report. Only BST data that has met the DQOs described in this QAPP will be reported and included in the final project report. Data and information produced thru this project will provide needed information pertaining to watershed characteristics, potential sources of pollution and will aid in the selection of BMPs to address identified water quality issues. Ultimately, stakeholders will use the information produced by this project for the development of appropriate measures to address water quality concerns in the study area. Information produced by this project will be used (through TSSWCB project 08-54) for watershed decisions regarding the development of a use attainability analysis or a total maximum daily load or a watershed protection plan.

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APPENDIX A. CORRECTIVE ACTION REPORT

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Corrective Action Report

CAR #:_____

Date:_____ Area/Location:_____

Reported by:_____ Activity:_____

State the nature of the problem, nonconformance, or out-of-control situation:

_____
_____
_____
_____

Possible causes:

_____
_____
_____

Recommended corrective action:

_____
_____
_____

CAR routed to:_____

Received by:_____

Corrective Actions taken:

_____
_____
_____
_____

Has problem been corrected? YES NO

Immediate Supervisor:_____

Project Leader:_____

Quality Assurance Officer:_____

TSSWCB:_____

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APPENDIX B. STANDARD OPERATING PROCEDURES

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B-1: Archival of *Escherichia coli* Isolates

Note: All handling of cultures will be performed using a Class 2 biological safety cabinet to minimize the exposure of laboratory personnel to pathogens. These isolates should be from colonies which have been plated for purity several times and lab personnel are confident purity has been achieved.

1. Select a well-isolated colony of purified *E. coli*. (Examine the cultures using a long-wave handheld UV lamp, colonies will fluoresce).

2. Using a bacteriological loop, transfer the colony to a labeled sterile cryovial containing 1 mL of tryptone soy broth (TSB) with 20% reagent grade glycerol.

3. Firmly cap the cryovial and verify that the cells have been resuspended by vortexing for several seconds; then plunge into liquid nitrogen until frozen.

4. Immediately transfer to a cryostorage box and place in -70 to -80°C freezer. Cultures may be stored for several years under these conditions.

5. To recover cultures from frozen storage, remove the cultures from the freezer and place the cryovials in a freezer block. *Do not allow cultures to thaw.*

    a. Using a bacteriological loop, scrape the topmost portion of the culture and transfer to growth medium, being careful not to contaminate the top or inside of the vial. Invert and incubate plates at 35 to 37°C for 20 to 24 h.

    b. Reclose the cryovial before the contents thaw and return to the freezer.

Casarez, E. A.; Pillai, S. D.; Mott, J.; Vargas, M.; Dean, K.; Di Giovanni, G. D. (2007). "Direct comparison of four bacterial source tracking methods and a novel use of composite data sets." J. Appl. Microbiol. In press doi:10.1111/j.1365-2672.2006.03246.x.

B-2: ERIC-PCR of *Escherichia coli*

1. Select isolated colonies from overnight cultures of *E. coli* isolates on Brain-Heart Infusion (BHI) plates.

2. Transfer colonies using a 1 µL loop to a sterile microfuge tube containing 100 µL of sterile molecular grade water; vortex briefly to suspend cells.

3. Prepare sufficient PCR Master Mix for samples, including one blank per 10 samples to account for volume loss due to repeat pipetting. Prepare Master Mix for each sample as noted below. One full PCR batch on the MJ Research Cycler 48 well-plate will have 46 samples, *E. coli* QC101, and a no template control.

   ERIC-PCR Master Mix – 24 samples + 2 blanks, prepare X 2 for full 48-well plate

MASTER MIX	Amt (uL)	Final Calc	Final Units
dH2O	**819**		
10X PCR buffer I w Mg	**130**	1	X (1.5 mM)
20 mM dNTP	**13**	200	uM each
ERIC Primer Mix	**130**	600	nM each
BSA (30 mg/ml)	**65**	1.5	ug/uL
AmpliTaqGold (Units)	**13**	2.5	Units/rxn

4. Dispense 45 µl of Master Mix for each sample into the appropriate well of PCR plate.

5. Briefly vortex cell suspensions, then add 5 µl of each cell suspension to the appropriate PCR well.

6. Carefully seal plate using an adhesive PCR cover.

7. Load the plate into the thermal cycler and run under the "ERIC-PCR" program with the following cycling conditions:

   a. Initial denaturation at 95°C for 10 min

   b. 35 Cycles:
      i. Denaturation at 94°C for 30 sec
      ii. Annealing at 52°C for 1 min
      iii. Extension at 72°C for 5 min

   c. Final Extension at 72°C for 10 min

8. Store completed reactions at -20°C until analyzed by gel electrophoresis.

9. Prepare a 250 mL, 2% agarose gel using a 500 mL bottle. Add 250 mL of 1 X Tris/Borate/EDTA (TBE) buffer and 5.0 g agarose. Microwave until agarose is fully

dissolved, tighten cap and let cool 1 to 2 minutes, then pour agarose into casting tray with 30-tooth, 1 mm thick comb.

10. Allow gel to solidify for approximately 30 minutes on the bench, then without removing comb place in Ziploc bag and solidify overnight in the refrigerator. The next day carefully remove comb, transfer to gel tank containing pre-cooled 1X TBE buffer. Replace TBE in gel tank after it has been used twice.

11. The following items will be needed for electrophoresis:

    100 bp ladder (0.33 µg/10 µL) (1500 µL final, enough for 150 lanes)

    200 µL Roche DNA Marker XIV (Cat. #1721933) 0.25 µg/µL 100 bp ladder (add reagents below to a full tube of marker)

    300 µL 6X ERIC-PCR loading buffer (see recipe below)

    150 µL 10X PCR buffer

    850 µL molecular grade water

    Store in cold room

    6X ERIC-PCR Loading Buffer

    25 mg bromphenol blue (0.25%)

    1.5 g ficoll 400 (15%)

    Add molecular grade water to 10 mL, divide into 1 mL aliquots and freeze, the aliquot currently being used can be stored in the cold room

    ERIC-PCR Blank

    100 µL 10X PCR buffer

    200 µL 6X ERIC-PCR loading buffer

    900 µL molecular grade water

    Store in cold room

    Ethidium Bromide Stain (0.5 µg/mL)

    1250 mL 1X TBE

62.5 µL ethidium bromide (Sigma, 10 mg/mL)

Store covered at room temp, can use up to 5 times by adding 10 µL ethidium bromide each additional use

12. Mix 10 µL of 6X ERIC-PCR Loading Buffer to each PCR well and mix with pipette tip.

13. Load the gel in the cold room as follows (max. of 23 samples + QC101 + NTC per gel):

    a. Load 10 µl of 100 bp ladder (0.33 µg) into the first lane

    b. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes

    c. Load 10 µl of 100 bp ladder (0.33 µg)

    d. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes

    e. Load 10 µl of 100 bp ladder (0.33 µg)

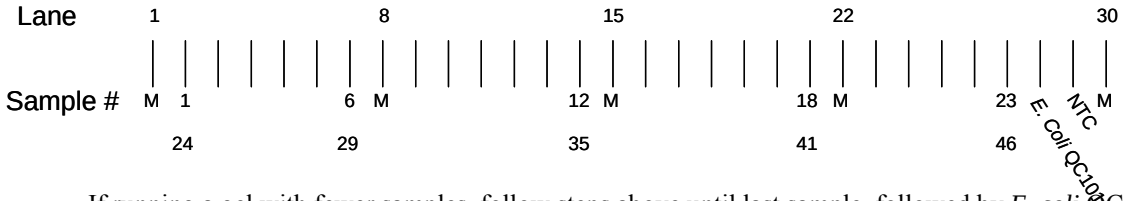
    f. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes

    g. Load 10 µl of 100 bp ladder (0.33 µg)

    h. Load 10 µl of sample ERIC-PCR reactions into next 5 lanes

    i. Load PCR Batch *E. coli* QC101 and NTC into next 2 lanes

    j. Load 10 µl of 100 bp ladder (0.33 µg)



If running a gel with fewer samples, follow steps above until last sample, followed by *E. coli* QC101, NTC and ladder, then load ERIC-PCR Blank into remaining lanes on gel.

14. Start electrophoresis power supply set at 100 volts, run for 1 hour.

15. Stop power supply, set time to "000", set voltage to 200 and start circulating pump at setting #2, run for 4 hours.

16. After electrophoresis, stain gel in Ethidium Bromide Stain for 20 minutes with agitation (save stain, see Step 13).

17. Destain gel for 10 minutes in 1X TBE buffer. Save destain, can be used 3 times then discard.

18. Follow Gel Imager SOP for image capture. Save digital photograph as a TIFF file (default) and print a hardcopy for notebook.

Casarez, E. A.; Pillai, S. D.; Mott, J.; Vargas, M.; Dean, K.; Di Giovanni, G. D. (2007). "Direct comparison of four bacterial source tracking methods and a novel use of composite data sets." J. Appl. Microbiol. In press doi:10.1111/j.1365-2672.2006.03246.x.

B-3: RiboPrinting of *Escherichia coli*

Storing and Handling Disposables

Check the lot expiration date on each label for details and rotate the stock to optimize use.

Heating membrane and probe (MP) Base

After storage and the temperature changes that occur during shipment, the oxygen in the buffer loaded in the MP base may need to be removed before use. This is called degassing and is accomplished by heating the base pack overnight in your incubator.

To degas buffer:

1. Place enough MP base packs for the next day's production in their storage pouches in an incubator set at 37°C.

2. Allow the base pack to degas for 16 to 24 hours prior to loading in the characterization unit. You may do this while you are incubating samples, since the base packs are sealed in their pouches. This procedure allows you to start a batch immediately at the beginning of the next shift.

3. If you do not use the heated base packs, you can return them to storage and reuse them. These base packs should be heated again before reuse since temperature cycling affects oxygen content in the buffer.

Preparing Lysing Agent (for *Staphylococcus* and lactic-acid bacteria only)

Lysing agent (A and B) is shipped frozen and must be stored at -20°C. Lysing agent must be thawed before use. This only takes about 5 minutes. If the lysing agent will not be used again for more than 2 hours, the material should be returned to the freezer. Lysing agent can be re-frozen several times with no effect on performance.

Sample Preparation Procedures

1. Incubate and Inspect the Samples

Use BHI agar plates prepared within the last 30 days. Do not use plates that appear dry or dehydrated. Such plates can cause problems when you attempt to "pick" the colonies for use in the RiboPrinter® system.

1. Using a pure isolated colony as the source, streak BHI agar plates heavily in the upper portion of the plate to create a lawn. Streak the remainder of the plate lightly to create single colonies.

2. Follow standard laboratory techniques. Heat plates for 18 to 30 hours in a humidified incubator at 37 °C.

2. Transfer Sample Buffer to Intermediate Tubes

1. Locate the 250 mL twist-top bottle of sample buffer supplied in Pack # 1. Install the twist cap.

2. Transfer about 5 mL of buffer to a sterilized disposable 15 mL intermediate working tube.

3. Add sample buffer to microcentrifuge tubes

1. Place a sterile 0.65 mL microfuge tube in each of the eight holes in the lower row of the sample preparation rack.

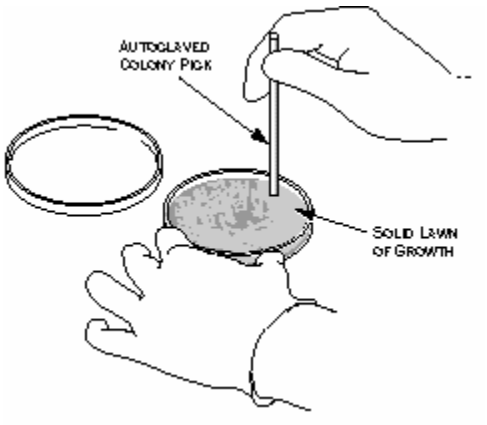
2. For Gram negative samples (including *E. coli*), add 200 µL of sample buffer from the intermediate tube.

   For Gram positive samples (e.g. *S. aureus* and *L. innocua* QC strains), add 40 µL of sample buffer.

3. Close the lids on the tubes.

4. Harvest the Samples

1. Using autoclaved colony picks and making certain not to gouge the agar, carefully place the pick into one of the single colonies or the lawn. You need a sample area at least equal to that of the bottom of the colony pick. In most cases you will need to harvest from the lawn area of the plate. If you are working with large colonies, a single colony will be adequate.



2. For Gram negative samples (e.g. *E. coli*), perform 1 pick placed into 200 µL of sample buffer.

CAUTION! Do not try to use the same pick twice on a plate. You need to harvest only enough sample to cover the bottom surface of the pick. Make sure the end of the pick is flat, if not, use a different pick.

CAUTION! Do not overload the harvesting pick. Collect only enough sample to cover the base of the pick. Over sampling will cause inaccurate results. Over sampling is a particular problem with *Staphylococcus*.

5. Mix the Samples

WARNING! *Perform sample preparation using a Class 2 biological safety cabinet since aerosols may be formed during mixing of the samples.*

1. Making certain not to touch the sample end of the pick, place the pick into one of the filled sample tubes.

2. While holding the tube with the open end facing away from you, carefully attach the pick to the hand-held mixer. The fit of the pick in the coupling will be loose.

WARNING! Do not turn on the mixer unless the pick is inside the sample tube and below the surface of the liquid. Turning the unit on at other times will cause the sample to aerosolize and may cause contamination.

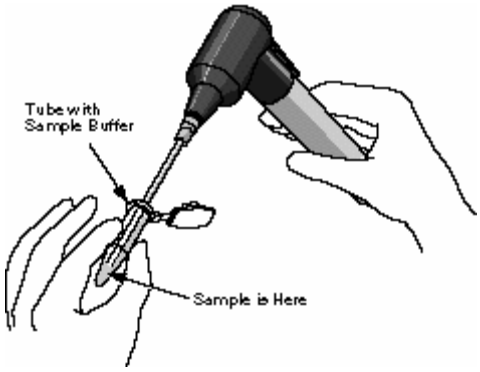
3. Press the ON lever on the mixer for about 5 seconds.

4. Release the lever and carefully remove the colony pick. The sample liquid should appear turbid.

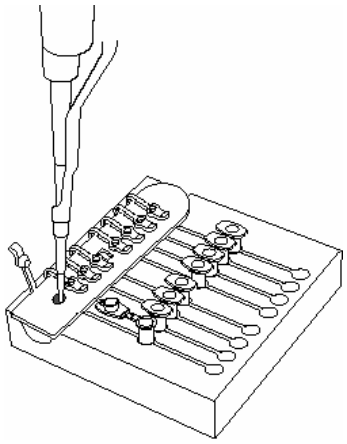
5. For **Gram positive samples only**, (e.g. *Staphylococcus* and *Listeria*) locate a new colony pick and repeat the steps for harvesting and mixing samples, adding a second sample to the original tube. Discard the used picks in a biowaste bag.

6. Cap the sample tube.

7. Move the tube to the top row of the sample preparation rack. This indicates that the tube is filled.



6. Transfer the Samples to the Sample Carrier



1. Open the lid covering the first well of the sample carrier.

2. Using a 100 µL pipetter, pipette 30 µL of sample from the microcentrifuge tube into the well.

3. Close the lid cover for the well.

4. Repeat for remaining samples using a new pipet tip for each sample.

CAUTION! Transfer the sample carrier to the Heat Treatment Station within 2 hours. If you wait longer than 2 hours, you will have to discard the sample carrier and begin again for this batch.

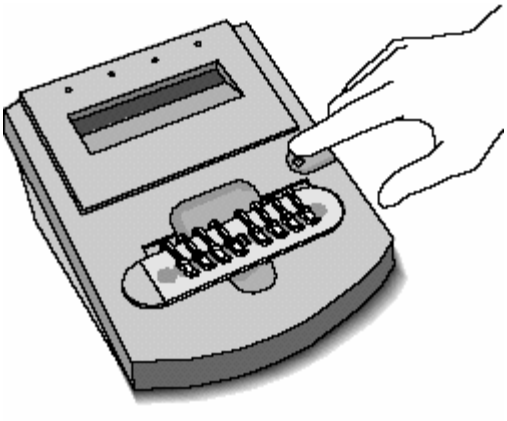
6. Lightly wipe down the outer surfaces of the sample carrier with a lab wipe wetted with surface disinfectant (10% bleach or 70% alcohol).

7. Write down the name or code you use to identify the sample and the well number in the sample carrier for each sample using a sample log sheet.

7. Place the Sample Carrier in the Heat Treatment Station and Process the Sample Carrier

1. Place the sample carrier into the Heat Treatment Station. The display on the Heat Treatment Station will show **Insert**, if power is available. If the display is blank, make certain that the power cord on the back of the station is properly connected.

   After you insert the carrier, the display shows **Press Button.**



2. Press the button on the Heat Treatment Station.

   The display shows **Warm up** and counts down from **10** while the station is warming up. The actual warm up cycle varies with the condition of the room and the heat treatment station. Normal time is about 4 minutes.

   When the station reaches operating temperature, the display changes to **Heat** and counts down from **13.** This represents each minute of heat treatment.

   The indicator message changes to **Cool.** The display counts down from **9**, indicating the minutes remaining in the cooling cycle**.** If necessary, you can remove the carrier as soon as the **Cool** message appears.

3. The heat treatment step is finished when the display shows **READY** and counts down from **90.** The display will flash and an audible beep will sound three times. The alarm will then beep once every 10 minutes until the sample is removed or 90 minutes elapses.

Caution! The heat-treated samples must be used within the 90-minute period at room temperature or they must be discarded. The heat-treated samples may be stored at this point (prior to adding Lysis Agents, if required) for 1 week at 4 °C, or for several months at -70 °C.

8. Add the Lysing Agents (for *Staphylococcus* and lactic-acid bacteria only)

1. Using a 10-µL pipetter and new tips for each addition, add 5 µL of Lysing Agents A and B to each sample. Note: this step is omitted for *E. coli* as it has no effect on ribopatterns. Lysing Agents were specifically developed for *Staphylococcus* and Lactic-Acid bacteria samples.

Caution! This step must be performed just prior (within 10 minutes) of loading the samples into the RiboPrinter and starting the run.

Creating and Loading a Batch

There are three options under the Operations menu for creating standard batches:

- *Eco*RI batches (VCA)
- *Pst*I batches (VCB)
- *Pvu*II batches (VCC)

You can also create special batches:

- Restriction Enzyme Flexibility batches
- **Substitute Enzyme batches (including *Hin*d III)**

From the Instrument Control Base Window:

1. Move the pointer to Operations and click with the mouse button. The Operations menu appears.

2. Move the pointer to Create Substitute Enzyme Batch and click with the mouse button.

3. Use the View menu to remove any optional items you do not wish to fill in. The system requires at least Sample Type and RiboGroup Library information for each sample. You cannot remove these options. The **Clear** option de-selects the **Use Default ID Libraries.** You will have to enter a DuPont ID and Custom ID library name for all samples. These become required fields and the system will make you enter data before you can save the information in this window.

CAUTION! If you change the display after you have entered information, you will lose all the information in the window. The window will redraw with a new blank display showing the items you have selected.

4. To enter information about the sample, click on the **View** button with the mouse button, then click on **Sample Items**. Click on the options you want to display.

5. Enter your initials and any comment you want to record about the batch.

6. Select the lot number fields and record for all reagents.

CAUTION! All fields must be completed or the system will not let you start processing the batch.

7. For each well in the sample carrier, choose the type (Sample or Control [QC Number]) from the Sample Type field. The system defaults to Sample.

8. Once you define the Sample Type as Sample, type in the name you actually want to use. This information will appear as Sample Label in the Data Analysis software screens.

9. You can change the RiboGroup library name if needed. Do this by clicking on the button next to the field with the mouse button. A pop up menu appears listing your choices. If you want to add a new library name, move the pointer to the line and click with the mouse button to get a cursor, then type in the new library name. Once you have saved this file, the new name will be added to the pop up list for future use. Do **NOT** change the DuPont ID field. If you select one of the QC strains, the system automatically enters QC in the DuPont ID and RiboGroup Library fields. Do not change these names. If you wish, you may enter a name for the Custom ID library.

10. Repeat for the other seven samples.

11. Click on Save and Submit Batch to Instrument.

Loading Disposables

Follow the screen prompts to load disposables and check the DNA Prep Waste. The icons on the window will flash red to tell you to remove and load an item. The screen prompts you about which Separation and Transfer chamber to use for the membrane and gel cassette. The LDD Pipette will move to physically block you from placing samples in the wrong chamber.

CAUTION! *Do not try to move the pipette manually. You will cause the system to lose the step count. This can result in the loss of batch data. If the pipette is blocking the S/T chamber that you are instructed to use, STOP. Call Customer Support.*

CAUTION! *Do not load disposables until you are prompted by the system. If you try to load them earlier, the alarm will sound as long as the doors are open. If you do load disposables ahead of time, the MP Base will be moved to the wrong position and you will not be able to begin processing the batch. You will not be able to move the MP base manually.*

1. Check the DNA Preparation Waste Container

1. The DNA Prep waste container must be visually checked before every batch. If the container looks nearly full (about 1 inch from the top), remove the container, unscrew the cap and empty into the liquid biohazard waste.

WARNING! Do not tip the DNA Preparation waste container when you remove it.

WARNING! Do not unscrew the cap from the DNA Preparation waste container if the fluid level has risen into the cap. First pour the excess waste liquid into the liquid biohazard waste.
WARNING! When replacing container make sure that the cap is properly threaded in place. If the cap is only partially threaded, it can snag the pipette during operation.

2. Load the Sample Carrier

1. Place the sealed carrier into the labeled slot on the far right of the characterization unit.

2. Push the sample carrier down firmly until it snaps into place.

CAUTION! Place the rounded edge of the sample carrier on your right as you view the characterization unit. Position the carrier this way to insure correct identification of the sample wells.

3. Load the DNA Prep Carrier

1. Remove the DNA Prep carrier from the refrigerator.

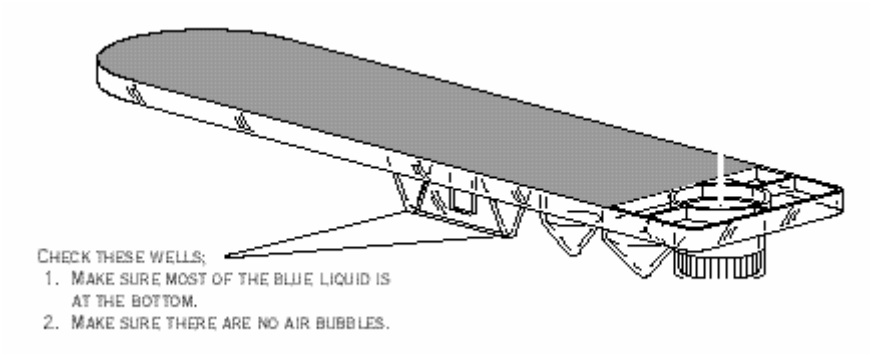
2. Check the wells in the carrier. If most of the liquid appears to be in the bottom of the wells and there are no bubbles, go to step 3. Otherwise **lightly tap the side of the carrier a few times with your finger to release any material that has adhered to the lid.**

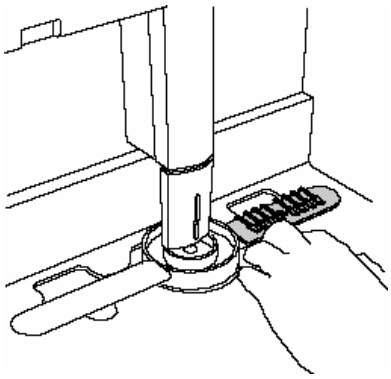
3. CAUTION! Do not tap the carrier briskly. This may cause the marker to degrade which can create inaccurate results.

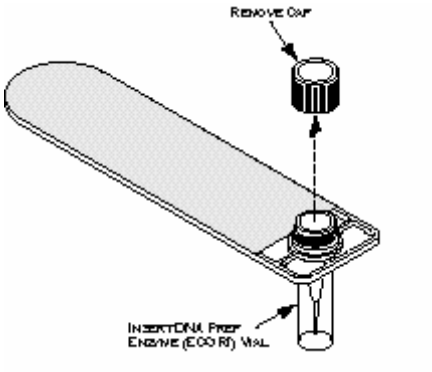
4. Remove a vial of DNA Prep Enzyme (*Hin*d III or *Eco*R I) from the freezer. ***Hin*d III (NEB Cat. #R0104M) is prepared in a Sarstedt 500-µL microfuge tube (Cat. #72730-005) as a 50 U/µL working stock as follows.**

   50 U/µL: 26.5 µL *Hin*d III and 26.5 µL of NEB 10X Buffer 2

   During addition of the Buffer, mix enzyme and buffer to homogeneity by stirring with the micropipette tip.





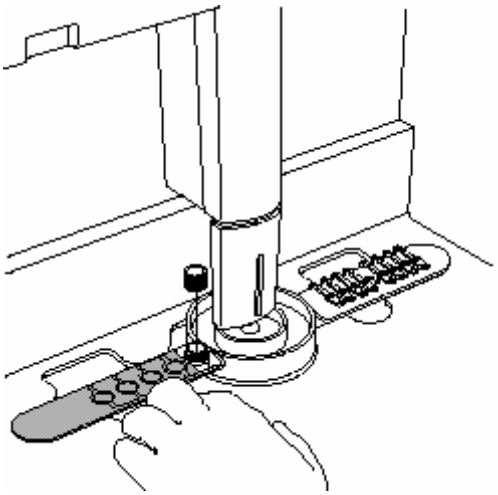


5. Remove the cap from the Enzyme vial.

6. Insert the vial into the carrier.

7. Place the DNA Prep carrier into the slot labeled **Reagent** to the left of the sample carrier slot.

8. Push the DNA Prep carrier down firmly until it snaps into place.

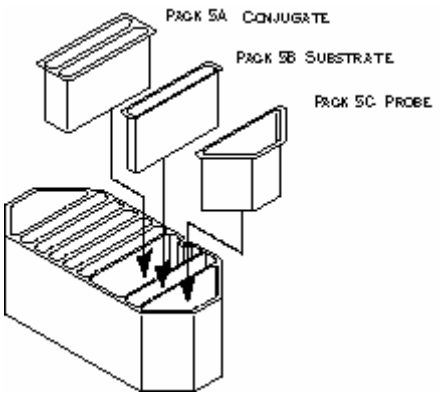


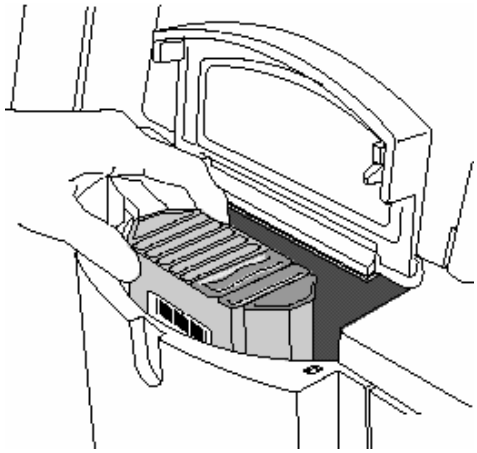
4. Load the MP Base and Carousel

1. Unpack the disposables.

2. Remove the MP base (Pack 5) from the incubator and the Conjugate (Pack 5A), Substrate (Pack 5B), and Probe (Pack 5C) from the refrigerator.

3. Remove each insert from its pouch. Tap the powdered reagent packs gently to bring all powder to the bottom of the packs. Place reagent packs in the MP base and load the base in the carousel.

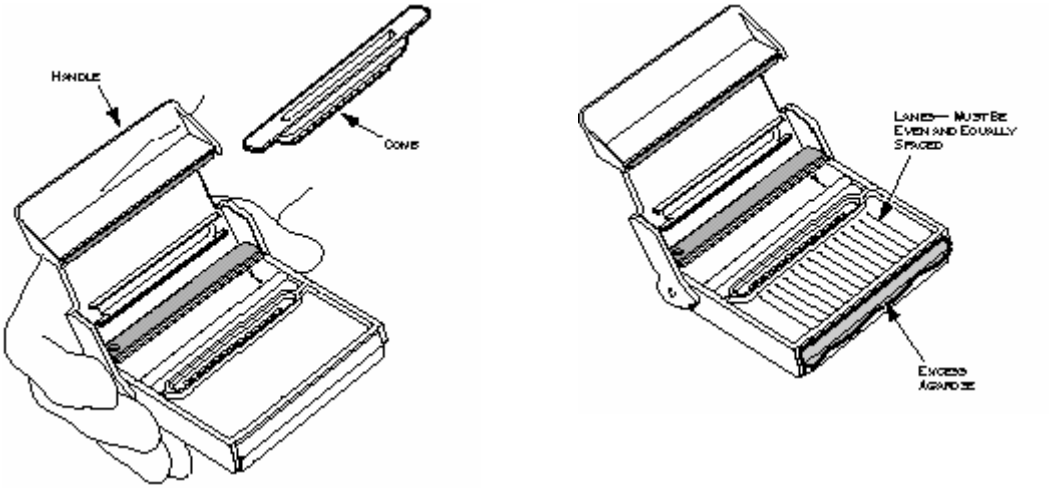




CAUTION! Push each insert firmly into place. If part of the insert extends above the top of the base, it could catch on the bottom of the deck and cause a system error. You could lose one or more batches as a result. Each insert is keyed by shape and cannot be inserted incorrectly.

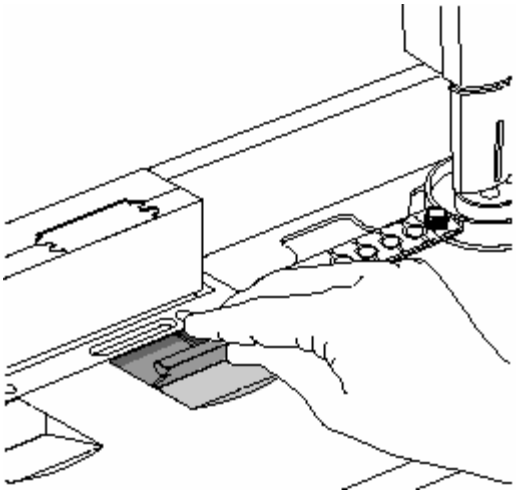
5. Load the Gel Cassette

1. Remove the gel cassette from its package.
2. Grasp one end of the rubber comb and gently pull the comb from the cassette.
3. Unfold the handle of the cassette towards you until the handle snaps into place.
4. Check the front edge of the gel cassette and the lanes of the gel.



Warning! *If the cassette shows a build up of excess gel on the front edge, or if you notice any shrinkage of the gel away from the cassette or bubbles, record the lot number and call Customer Support. Use a new cassette for this run.*

5. Insert the gel cassette into the slot labeled **Gel Bay.** The RiboPrinter® system will prevent the insertion of the cassette into the incorrect slot by blocking one slot with the LDD Pipette.

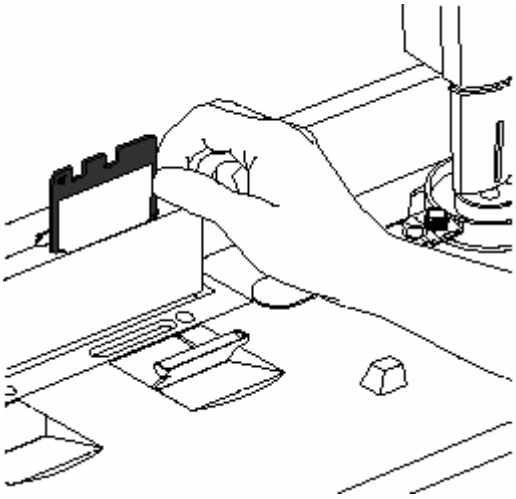


6. Press the cassette forward firmly until it snaps into place.

6. Load the Membrane

1. Grasp the membrane and carefully drop it into the front slot and flip the metal bracket against the back of the membrane.

CAUTION! You can insert the membrane backwards. This will cause an alarm that prevents the sample from being processed until the error is corrected. Always make certain that the two large slots are on top and that the square hole on the side faces your left as you insert the membrane.



7. Close all doors and the instrument will begin sample processing.

8. Load the Next Batch

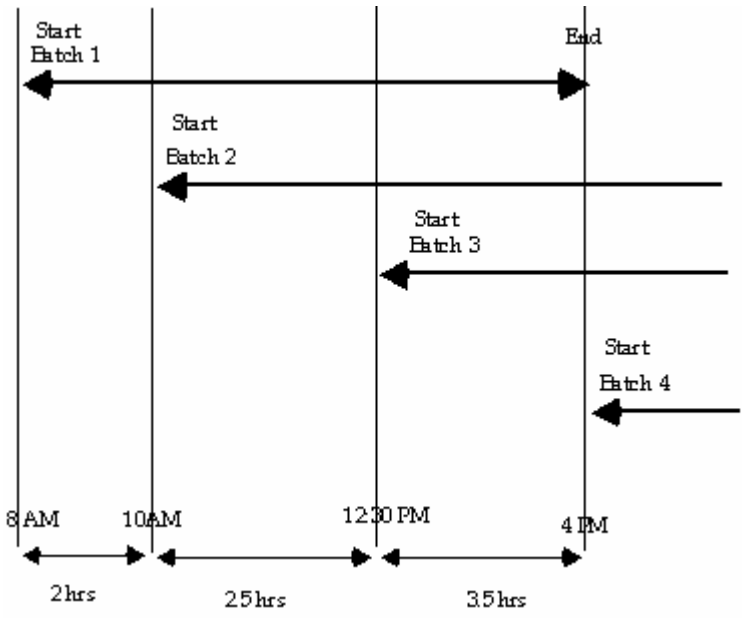
The RiboPrinter® microbial characterization system lets you load up to four VCA batches in an eight hour period. Other batches may take longer to process.
The chart above shows the approximate loading times for each batch in a work shift using only the VCA protocol.

1. You can now use the **Create Batch** option to set up a new pending batch.

2. When you complete the information window and click on the **Start Normal Batch** option, the window displays a message telling you when you can load the next batch.

Batch Report

After image processing is completed, the system automatically runs a series of analysis functions and generates a Batch Information Report. This task does not require any action on the part of the operator. Reports are automatically saved to the hard disk of the computer and sent to the printer.



Casarez, E. A.; Pillai, S. D.; Mott, J.; Vargas, M.; Dean, K.; Di Giovanni, G. D. (2007). "Direct comparison of four bacterial source tracking methods and a novel use of composite data sets." J. Appl. Microbiol. In press doi:10.1111/j.1365-2672.2006.03246.x.

B-4: *Bacteroidales* PCR

Preprocessing of Water Samples

1. Within six hours of sample collection, water samples (100 ml) are filtered through 0.2 µm pore size Supor-200 filters (VWR cat # 28147-979). Discard filtrate and place the filter into a pre-labeled sterile 15 ml tube (VWR cat# 21008-103) using ethanol-flamed forceps and aseptic technique. If 100 ml of water cannot be filtered, record the volume filtered on the 15 ml tube and COC.

2. Add 500 µl of guanidine isothiocyanate (GITC) lysis buffer to each 15 ml tube with filter.

   100 ml of GITC lysis buffer
   50 ml reagent grade (deionized) water
   59.08 g GITC (VWR # 100514-046; 5 M final)
   3.7 g EDTA [pH 8.0] (VWR # VW1474-01; 100 mM final)
   0.5 g Sarkosyl (VWR # 200026-724; 0.5% final)
   Adjust to pH 8.0 with NaOH (approx. 0.4 g of pellets) to dissolve EDTA and heat with vigorous stirring to dissolve guanidine
   Bring up to 100 ml total volume with reagent grade (deionized) water
   Autoclave and store at room temp

3. Store samples at -80°C (or -20°C manual defrost freezer, not the standard auto-defrost).

4. DNA will be extracted from the samples and analyzed by *Bacteroidales* PCR as described below.

DNA Extraction and PCR

1. DNA is extracted from the water concentrates using QIAamp DNA mini kit. Turn on the slide warmer and set to maximum. Preheat a microfuge tube rack and 0.01X TE buffer pH 8.0 for elution and a 70°C water bath.

2. Add 500 µl of Buffer AL to each thawed tube and vigorously agitate for 1 min using a wrist action shaker.

3. Incubate in a 70°C water bath for 10 minutes.

4. Transfer lysate to a 2.0 ml microfuge tube.

5. Add 500 µl of 100% ethanol and pulse vortex mix for 15 sec. Quick spin to remove droplets from cap.

6. Transfer half of the sample lysate (600 to 750 µl) to a labeled QIAamp column placed in a Qiagen collection tube. Microfuge at 14K rpm, with brake, for 1 minute. If necessary, at each step wipe off any buffer from outside of column with a lab tissue before placing into a new collection tube.

7. Place column in a new collection tube and repeat Step 6 with the remaining sample.

8. Place column in new collection tube and add 500 µl of AW1 wash buffer. Centrifuge as above and place column in a new collection tube.

9. Add 500 µl of AW2 wash buffer and centrifuge as above, then repeat once more. Place column in a clean collection tube and centrifuge as above to remove all traces of AW2 buffer.

10. Place in a clean collection tube in the heated rack on the slide warmer. Add 100 µl of 70 to 80 °C 0.01X TE buffer pH 8.0 and let incubate at 70 to 80 °C for 5 minutes with columns capped.

11. Immediately centrifuge at 14K rpm for 3 minutes and transfer the filtrate containing the eluted DNA to a labeled 0.65 ml tube. Store at -80 °C until analyzed by PCR. Keep the remainder of the unused aliquot of 0.01X TE to use as a no template control for the PCR.

Bacteroidales PCR Master Mix

1. Prepare sufficient PCR Master Mix for samples and controls, as well as one blank per 10 samples to account for volume loss due to repeat pipetting.

Bacteroidales **PCR Master Mix – per sample**

MASTER MIX	Amt (uL)	Final Calc	Final Units
Molecular Grade Water	**30.2**		
10X PCR buffer I w Mg (ABI)	**5**	1	X
MgCl2 (25 mM) (ABI)	**1**	0.5 (2.0 final)	mM
each dGTP, dCTP, dATP (33 mM mix) (Amersham)	**0.3**	200	uM each
dUTP (100 mM) (Amersham)	**0.2**	400	uM
Bacteroidales Primer Mix	**5**	200	nM each
BSA (30 mg/mL)	**2.5**	1.5	ug/uL
AmpliTaqGold (Units)	**0.5**	2.5	Units/rxn
Uracil DNA glycosylase NEB (UDG; 1 U/rxn)	**0.25**	0.5	Units/rxn

2. Dispense 45 µl of Master Mix for each sample into the appropriate well of PCR plate.

3. Briefly vortex DNA extracts, quick spin, then add 5 µl to the appropriate PCR well.

4. Carefully seal plate using an adhesive PCR cover.

5. Load the plate into the thermal cycler and run under the appropriate *Bacteroidales* program with the following cycling conditions:

    a. UDG digestion 50°C for 10 min

    b. Initial denaturation at 95°C for 10 min

    c. 40 Cycles:
        i. Denaturation at 95°C for 30 sec
        ii. Annealing at 53°C to 62°C (depending on primer set) for 1 min
        iii. Extension at 72°C for 1 min

    d. Final Extension at 72°C for 10 min

6. Store completed reactions at -20°C until analyzed by gel electrophoresis.
7. Prepare a 200 mL, 2% agarose gel using a 500 mL bottle. Add 200 mL of 1 X TBE buffer and 4.0 g agarose. Microwave until agarose is fully dissolved, add 10 µl of ethidium bromide (10 mg/ml), tighten cap, swirl to mix and let cool 1-2 minutes.

8. Pour agarose into casting tray with one or two 20-tooth, 0.75 mm thick combs.

9. Allow gel to solidify for 30-60 minutes on the bench, remove comb(s), and place in gel tank with TBE buffer. Discard TBE in gel tank after it has been used twice.

10. The following items will be needed for electrophoresis:

    <u>100 bp ladder (0.33 µg/10 µL) (1500 µL final, enough for 150 lanes)</u>

    200 µL Roche DNA Marker XIV (Cat. #1721933) 0.25 µg/µL 100 bp ladder (add reagents below to a full tube of marker)

    300 µL 6X Loading Buffer (see recipe below)

    150 µL 10X PCR buffer

    850 µL molecular grade water

    Store in cold room

    <u>6X Loading Buffer</u>

    25 mg bromphenol blue (0.25%)

    1.5 g ficoll 400 (15%)

    Add molecular grade water to 10 mL, divide into 1 mL aliquots and freeze, the aliquot currently being used can be stored in the cold room

11. Mix 10 µl of PCR product with 2 µl of 6X Loading Buffer in the appropriate well of a Nunc Module.

12. Load the gel, starting with 10 µl of 100 bp ladder in the first lane, followed by 12 µl of each sample with Loading Buffer, and 10 µl of 100 bp ladder after the last sample.

13. Start electrophoresis power supply set at 100 volts, run for 1.5 hours.

14. Follow Gel Imager SOP for image capture. Save digital photograph as an 8-bit TIFF file with no scaling and print a hardcopy for notebook.

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APPENDIX C. CHAIN OF CUSTODY FORM

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BRAZOS RIVER AUTHORITY CHAIN-OF-CUSTODY (please print)

Project Manager/Person(s) Requesting Sample _____

Sample Number	Case No. or Site/Location Description	Date of Collection	Time of Collection	Collector Initials	Preservation	Sample Type	Sample Matrix	Analysis Requested	Comments
1.									
2.									
3.									
4.									
5.									
6.									
7.									
8.									
9.									
10.									
11.									
12.									
13.									
14.									
15.									

Preservation: **I**=Ice, **A**=Acid, **O**=Other (Explain)_____

Sample Type: **G**=Grab, **C**=Composite, **O**=Other (Explain)_____

Sample Matrix: **S**=Surface Water, **W**=Waste Water, **D**=Drinking Water, **S**=Soil, **O**=Other (Explain)_____

Analysis: **TSS**, **TDS**, **Anions** (Cl, SO4, NO3-N, OPO4-P), **Chl *a***, ***E. coli***, Other (Explain)_____

Relinquished By (Print): _____ (Signature) _____ Date/Time:_____

Received By (Print): _____ (Signature) _____

Title: _____ Date/Time:_____

Comments:

Received By (Print): _____ (Signature) _____

Title: _____ Date/Time:_____

Comments: