Texas Nonpoint Source Grant Program

Statewide Bacterial Source Tracking Program for FYs 2013-2014

TSSWCB Project 13-50

Revision #1

Quality Assurance Project Plan

Texas State Soil and Water Conservation Board

Prepared by:

Texas Water Resources Institute

The University of Texas Health Science Center at Houston School of Public Health, El Paso Regional Campus

Texas A&M AgriLife Research Soil and Aquatic Microbiology Lab

Texas A&M Institute of Renewable Natural Resources

Effective Period: Upon TSSWCB Approval through May 31, 2015

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

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Section A1: Approval Sheet

Quality Assurance Project Plan (QAPP) for the *Statewide Bacterial Source Tracking Program for FYs 2013-2014.*

Texas State Soil and Water Conservation Board (TSSWCB)

Name: Jana Lloyd
Title: TSSWCB Project Manager (PM)

Signature:_____ Date: _____

Name: Mitch Conine
Title: TSSWCB Quality Assurance Officer (QAO)

Signature:_____ Date: _____

Texas A&M AgriLife Research – Texas Water Resources Institute (TWRI)

Name: Kevin Wagner, PhD
Title: TWRI Associate Director; Project Lead

Signature:_____ Date:_____

Name: Lucas Gregory
Title: TWRI QAO

Signature:_____ Date: _____

University of Texas Health Science Center at Houston School of Public Health, El Paso Regional Campus (UTSPH-EP)

Name: George D. Di Giovanni, PhD
Title: Prof. of Environ. & Occupational Health Sci.; UTSPH-EP Project Co-Lead

Signature:_____ Date: _____

Texas A&M AgriLife Research - Soil and Aquatic Microbiology Lab (SAML)

Name: Terry Gentry, PhD
Title: Assoc. Prof. of Soil & Aquatic Microbiology; SAML Director & Project Co-Lead

Signature:_____Date: _____

Texas A&M Institute of Renewable Natural Resources (IRNR)

Name: Roel Lopez, PhD
Title: IRNR Director; IRNR Project Co-Lead

Signature:_____Date:_____

Section A2: Table of Contents

List of Acronyms and Abbreviations

BMP	best management practice
BST	Bacterial source tracking
CAR	corrective action report
CFU	colony forming unit
COC	chain of custody
CSU	carbon source utilization
C_T	threshold cycle
DQO	data quality objectives
DNA	Deoxyribonucleic acid
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction
ERIC-RP	ERIC-PCR and RiboPrinting combination method
E. coli	*Escherichia coli*
IRNR	Texas A&M Institute of Renewable Natural Resources
KB-ARA	Kirby-Bauer antibiotic resistance analysis
MS Excel	Microsoft Excel
mRNA	Messenger ribonucleic acid
mTEC	membrane thermotolerant *E. coli*
MUG	4-methylumbelliferyl-β-D-glucuronide
NA-MUG	nutrient agar with MUG
NIST	National Institute of Standards and Technology
NPS	nonpoint sources (of pollution)
PCR	polymerase chain reaction
PM	Project Manager
QA	quality assurance
QAPP	quality assurance project plan
QAO	Quality Assurance Officer
QC	quality control
QPR	quarterly progress report
R&D	Research and Development
RPD	Relative percent deviation
RP	Riboprinting
RSD	relative standard deviation
SAML	Texas A&M AgriLife Research- Soil and Aquatic Microbiology Lab
SOP	Standard operating procedure
spp	species
TCEQ	Texas Commission on Environmental Quality
TMDL	total maximum daily load
TSSWCB	Texas State Soil and Water Conservation Board
TWRI	Texas A&M AgriLife Research, Texas Water Resources Institute
USEPA	United States Environmental Protection Agency
UTSPH-EP	University of Texas School of Public Health, El Paso
UV	ultraviolet
WPP	Watershed protection plan

Section 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
P.O. Box 658; Temple, Texas 76503

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Name: Roel Lopez, PhD
Title: IRNR Director; IRNR Project Co-Lead

Section A4: Project/Task Organization

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

TSSWCB – Texas State Soil and Water Conservation Board, Temple, Texas. Provides project overview at the State level.

Jana Lloyd, TSSWCB PM

Responsible for ensuring that the project delivers data of known quality, quantity, and type on schedule to achieve project objectives. Tracks and reviews deliverables to ensure that tasks in the work plan are completed as specified. Reviews and approves QAPP and any amendments or revisions and ensures distribution of approved/revised QAPPs to TSSWCB participants.

Mitch Conine; TSSWCB QAO

Reviews and approves QAPP and any amendments or revisions. Responsible for verifying that the QAPP is followed by project participants. Monitors implementation of corrective actions. Coordinates or conducts audits of field and laboratory systems and procedures. Determines that the project meets the requirements for planning, quality assurance (QA), quality control (QC), and reporting under the Texas Nonpoint Source Program.

TWRI – Texas Water Resources Institute, College Station, Texas. Responsible for general project oversight, coordination administration, reporting and development of data quality objectives (DQOs) and a QAPP.

Kevin Wagner, TWRI Associate Director; Project Lead

The TWRI 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 project QAPP; assessing the quality of subcontractor/participant work; and submitting accurate and timely deliverables to the TSSWCB PM. Responsible for supporting the development and ensuring the timely delivery of project deliverables, ensuring cooperation between project partners, providing fiscal oversight and completing project reporting.

Lucas Gregory, TWRI QAO

Responsible for determining that the QAPP meets the requirements for planning, QA and QC. Conducts audits of field and laboratory systems and procedures. Responsible for maintaining the official, approved QAPP, as well as conducting quality assurance audits in conjunction with TSSWCB personnel.

UTSPH-EP – University of Texas Health Science Center at Houston, School of Public Health, El Paso Regional Campus, El Paso, Texas. Responsible for bacterial source tracking.

George D. Di Giovanni, Prof., Environ. & Occup. Health Sci.; UTSPH-EP Project Co-Lead

Responsible for performing BST analysis and related activities. This includes 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 action is 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.

SAML – Texas A&M AgriLife Research – Soil and Aquatic Microbiology Lab, College Station, Texas. Responsible for bacterial source tracking.

Terry Gentry, Assoc. Prof. of Soil & Aquatic Micro.; SAML Director & Project Co-Lead

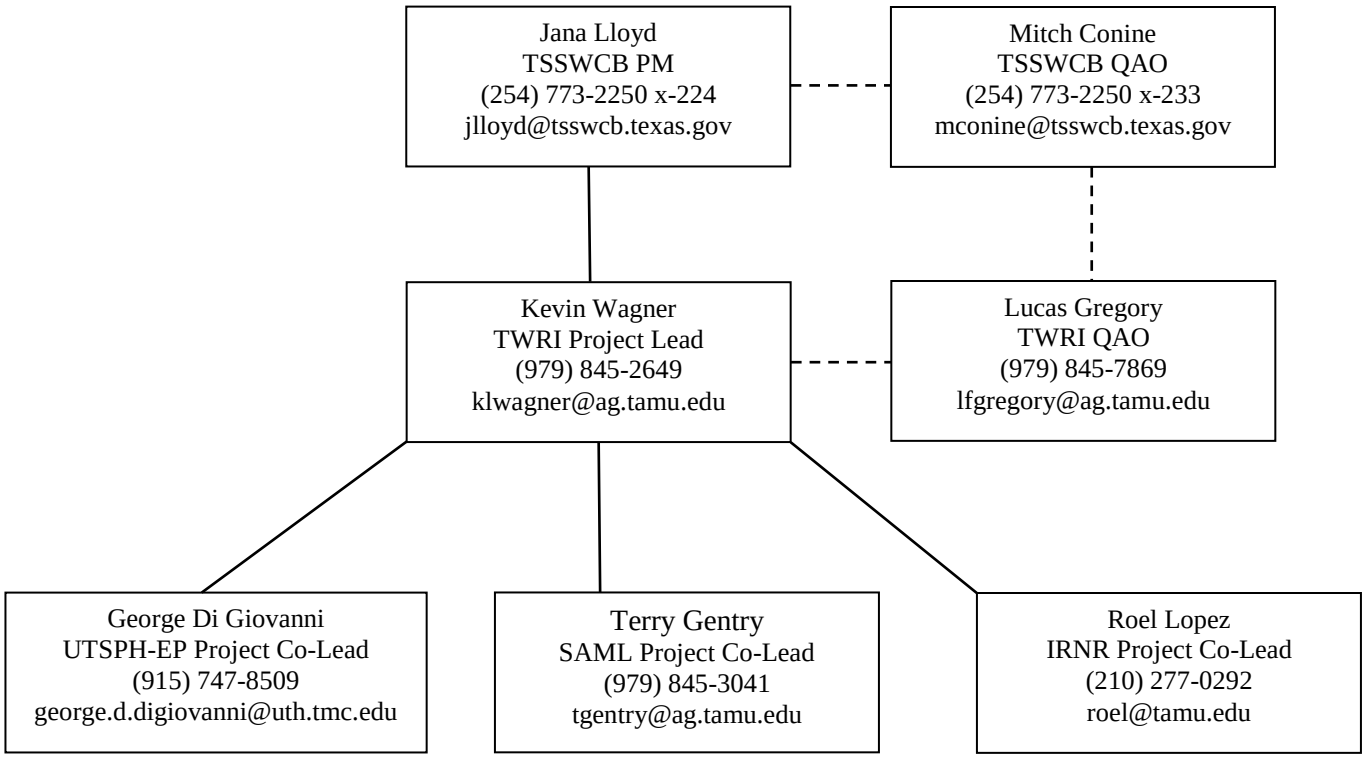
Responsible for performing BST analysis and related activities. This includes ensuring that SAML personnel involved in generating analytical data have adequate training and thorough knowledge of the QAPP and all SOPs specific to the analyses or task performed. Responsible for oversight of all SAML operations 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 action is implemented, documented, reported and verified. Monitors implementation of the measures within SAML to ensure complete compliance with project DQOs in the QAPP. Conducts in-house audits to ensure compliance with written SOPs and identify potential problems.

IRNR – Texas A&M Institute of Renewable Natural Resources, San Antonio and Gatesville, Texas. Responsible for fecal sample collection.

Roel Lopez, IRNR Director; IRNR Project Co-Lead

Responsible for overseeing fecal sample collection activities. This includes ensuring that field personnel involved in sample collection have adequate training and thorough knowledge of the QAPP and all SOPs specific to the task performed. Responsible for oversight of fecal sampling tasks ensuring that all QA/QC requirements are met, documentation related to collections is complete and adequately maintained and reported accurately. Responsible for ensuring that corrective action is implemented, documented, reported and verified. Monitors implementation of field measures to ensure complete compliance with project DQOs in the QAPP. Conducts field audits to ensure compliance with written SOPs and identify potential problems.

Figure A4.1 Project Organization Chart



Section A5: Problem Definition/Background

Protection of water resources is one of the most significant environmental challenges of the new millennium. Nonpoint sources (NPS) of pollution, including agricultural activities, can greatly impact water quality. One key component in effectively implementing a NPS pollution abatement program is the identification and assessment of sources of fecal pollution. Proper evaluation of these sources is needed to target best management practices (BMPs) and develop bacterial total maximum daily loads (TMDLs) or watershed protection plans (WPPs). This information may also be useful to properly assess risk in contact recreation, as many waterborne pathogens causing human illness do not colonize nonhuman hosts. According to the *2010 Texas Integrated Report*, there are over 300 impairments due to excessive bacteria.

Fecal coliform bacteria have extensively been used as an indicator of fecal pollution and the potential presence of other pathogenic microorganisms in water. It has been established that the fecal coliform bacterium *E. coli* is more closely associated with fecal pollution than other fecal coliform bacteria, which may normally reside and multiply in the environment. *E. coli* is a common inhabitant of animal and human intestines and recent studies have shown that isolates from humans and various host animals (e.g., cattle, chickens, and pigs) may differ genetically and phenotypically. Use of genetic and biochemical tests may allow the original host species to be identified and is referred to as bacterial source tracking (BST).

The premise behind BST is that genetic and phenotypic tests can identify bacterial strains that are host specific so that the original host species and source of the fecal contamination can be identified. Often *E. coli* or *Enterococcus* spp. are used as the bacteria targets in BST, as this provides a direct link with water quality standards which are usually based on one of these two indicators. While there has been some controversy concerning host specificity and survival of *E. coli* in the environment, this indicator organism has the advantage that it is known to correlate with the presence of fecal contamination and is used for human health risk assessments. BST of *E. coli*, therefore, has the advantages of direct regulatory significance and availability of standardized culturing techniques for water samples, such as USEPA Method 1603.

BST is a valuable tool for identifying human and animal sources of fecal pollution. Comprehensive BST has been completed by UTSPH-EP (formerly with Texas A&M AgriLife Research) for (1) the Lake Waco and Belton Lake watersheds, (2) several San Antonio area watersheds, (3) the Lake Granbury watershed, (4) Buck Creek, and (5) the Leon and Lampasas Rivers watersheds. The Waco/Belton and Buck Creek studies were funded by the TSSWCB through Clean Water Act §319(h) NPS grants from the U.S. Environmental Protection Agency (EPA) (TSSWCB projects 02-10 and 06-11, respectively) and the Leon and Lampasas project through state funds (TSSWCB project 10-51); while the San Antonio study and Lake Granbury studies were funded by the Texas Commission on Environmental Quality (TCEQ). In addition, SAML has completed BST projects for the Little Brazos River tributaries, and Big Cypress Creek watersheds (TSSWCB projects 09-52 and 09-55, respectively). Additionally, with TSSWCB funding, BST projects are currently under way in the Leona River and Attoyac Bayou watersheds to assess water quality impairments (projects 11-50 and 09-10, respectively).

A Texas *E. coli* BST Library has been developed based on known source isolates from the Waco/Belton, San Antonio, Granbury, Buck Creek, Big Cypress, Little Brazos River, Attoyac Bayou, Leon River, Lampasas River, Upper Trinity River and Upper Oyster Creek watersheds. The Texas *E. coli* BST Library (ver. 8-12) currently contains 1,669 *E. coli* isolates obtained from 1,455 different domestic sewage, wildlife, livestock and pet fecal samples. While this represents a significant step towards development of a statewide *E. coli* BST library, continued expansion of the library to include additional known source isolates from different Texas watersheds and different animal hosts is still needed. This will allow continued evaluation of the library for geographical stability and the diversity of source specific isolates to identify specific needs for future expansion and refinement of the library. The use of the Texas *E. coli* BST Library will provide for significant cost and time savings for the identification of NPS pollution in the development of TMDLs and WPPs.

A Task Force on Bacteria TMDLs was jointly established by the TSSWCB and the TCEQ in fall 2006. In the Task Force's Report, a strategy to address current and future bacterial TMDLs and Implementation Plans (I-Plans) was outlined. The Task Force describes and makes recommendations for effective use of BST methods that have been used in Texas. These include enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR), RiboPrinting (RP), Kirby-Bauer antibiotic resistance analysis (KB-ARA), carbon source utilization (CSU), and *Bacteroidales* PCR. The Task Force recommended using library-independent methods such as *Bacteroidales* PCR for preliminary qualitative analyses and library-dependent methods (e.g., ERIC-PCR and RP) if more quantitative data are required. Further characterization of known source *E. coli* for expansion of the Texas *E. coli* BST Library and continued support of established BST analytical infrastructure will help achieve the recommendations of the Task Force.

The Task Force Report identified certain Research and Development (R&D) needs to advance understanding of bacteria. Specifically, 30 types of studies or research needs in 6 categories (including Characterization of Sources and Bacterial Source Tracking) were identified. This list was not exhaustive and no attempt was made to prioritize these activities. As such, there is a need to update, expand and prioritize these BST-related R&D activities.

Lastly, the state of BST science, methodologies, application and confidence has evolved greatly in the past few years. A host of new information is currently available, yet not readily distributed or known to state and federal agency personnel. To address this, the *2012 BST – State of the Science Conference* was held. To build on the success of this conference, continued outreach and technology transfer is needed to foster dialogue and collaboration and bring water resource managers up to speed on advances in BST technologies, methodologies, applications and results.

Section A6: Project/Task Description

General Project Description

The goal of this project is to support BST analyses across the State through (1) maintenance of analytical infrastructure at public BST laboratories; (2) continued development and implementation of statewide BST template-SOPs; (3) delivery of informational materials on bacteria BMPs and the use and applicability of BST and the State-supported analytical labs; (4) further expansion and evaluation of the Texas *E. coli* BST Library; and (5) further development of suitable source-specific bacterial markers for library independent BST.

Maintenance of Analytical Infrastructure

Due to the current and anticipated need for BST studies in Texas, statewide BST analytical infrastructure needs to be maintained appropriately. This not only includes the needed maintenance and repairs of analytical equipment; but also the continued support, training, and retention of skilled personnel. To meet the needs of the State, BST analytical capabilities will be maintained at both UTSPH-EP and SAML BST laboratories. Training needs for each individual laboratory's personnel will be coordinated to ensure appropriate technology transfer and comparability of BST data.

Continued Development and Implementation of Statewide BST Template-SOPs

While previous studies have utilized appropriate QA and QC mechanisms as identified in project-specific QAPPs, the volume of current and anticipated BST studies across the State favors the development and implementation of BST template-SOPs. BST template-SOPs developed under TSSWCB projects 08-50 and 08-51 have provided for the continued development and use of the Texas *E. coli* BST Library by multiple laboratories and will also support and improve inter-laboratory comparison of BST results. In this project, ERIC-PCR, RP and *Bacteroidales* PCR template-SOPs will be reviewed and updated accordingly to ensure that they are current and up to date with applicable methods, technologies and markers.

Known-Source Sample Collection and Library Expansion

The Texas *E. coli* BST Library is dynamic, with new isolates being added with each successive BST project. The current library (ver. 8-12) contains known source isolates from over a dozen watersheds, as well as wildlife isolates from South Texas. While this represents a significant step towards development of a statewide *E. coli* BST library, continued expansion of the library to include additional known source isolates from different Texas watersheds and different animal hosts is still needed. In particular, the state library has very few *E. coli* from wildlife species such as mice, rabbits, nutria and squirrels and none from bats. Under this project, ERIC-RP data for approximately 100 known source *E. coli* isolates from the Leona River watershed (TSSWCB Project 11-50) will be provided by SAML to UTSPH-EP for analysis and expansion of the state library. In addition, approximately 100 known source fecal samples from targeted animal sources will be collected and the *E. coli* analyzed to further expand the state library and provide additional datasets for library evaluation. 20 known-source *E. coli* isolates from bats (TSSWCB

Project 11-51) will also be analyzed. Isolates will be added to the Texas *E. coli* BST Library utilizing the ERIC-PCR and RiboPrinting composite method. Isolates will be screened using ERIC-PCR and the non-clonal isolates will be further analyzed using RiboPrinting. By collecting some of these known-source samples from a previously studied watershed (e.g., Leon River watershed), the temporal stability of the library will also be assessed. The geographic stability of the library will be evaluated by performing watershed exclusive and inclusive statistical analyses. In addition, the fingerprint diversity of source-specific *E. coli* isolates will be investigated to help evaluate the strain representativeness of the library. This will allow the project team to identify specific needs for the future expansion and refinement of the library.

Methods Development

There have been significant developments in library-independent BST methods, including bacterial genetic markers specific to different animal sources and humans. Library-independent methods are cost-effective, rapid, and potentially more specific than library-dependent methods. Concerns with many of the recently developed library-independent approaches include uncertainties regarding geographical stability of markers and the difficulty of interpreting results in relation to regulatory water quality standards and microbial risk, since some target microorganisms are not regulated. More importantly, these library-independent methods can only detect a limited range of pollution sources and are currently only semi-quantitative. For example, the *Bacteroidales* PCR can detect fecal pollution from ruminants, humans, dogs, horses and pigs; but currently no further discrimination is possible. Despite these limitations, this method may be very useful for the rapid and inexpensive assessment of the possible sources of fecal pollution impacting a waterbody. UTSPH-EP (under TSSWCB project 10-50) has generated promising preliminary results for a *Bacteroidales* PCR method to detect feral hog fecal pollution, as well as identified possible genetic targets for discriminating human and animal *E. coli*. A simple library-independent method for distinguishing human from animal *E. coli* would be quite useful for BST studies. Current research in this area at UTSPH-EP is based on sequence analysis of ERIC-PCR products from isolates identified through data mining of the Texas *E. coli* BST Library. Library-independent source-specific methods have recently been described for poultry and cattle. Importantly, UTSPH-EP has observed some cross-reactivity of animal fecal DNA with *Bacteroidales* PCR markers, especially for the human HF183 marker. This occurred for some known source wildlife samples in the Buck Creek project (TSSWCB project 06-11) which were collected from a remote site which had very limited human access. This may explain the unexpected and frequent occurrence of water samples positive for the human marker at this site. To help explore the issue of cross-reactivity, all 100 known-source fecal samples collected under this project will be analyzed for the human HF183 marker. In addition, the *Brevibacterium avium*-based poultry marker developed by Weidhaas et al. (2010) has potential for use in Texas watersheds that receive applications of poultry litter/manure. SAML will evaluate this new marker using ≥100 known-source fecal samples and water samples collected under previous TSSWCB projects (09-52, 09-10, 09-05) and the current project. Further development and evaluation of these library-independent methods will be conducted for possible inclusion into Texas' BST toolbox.

Outreach on Bacterial Source Tracking and BMPs

In order to provide outreach on BST and BMPs, TWRI and the project team will maintain the project website; meet with natural resource agencies and researchers engaged in BST; distribute educational brochures; and participate in or host conferences, workshops, and/or seminars.

Table A6.1 Project Plan Milestones

Task	Project Milestones	Agency	Start	End
2.1	Develop QAPP	TWRI, IRNR, UTSPH-EP, SAML	2/13	7/13
2.2	Submit revisions/amendments to QAPP	TWRI	7/13	5/15
2.3	Updated BST template-SOPs for ERIC-PCR, RiboPrinting, and *Bacteroidales* PCR ensuring that template-SOPs include current methods, technologies and approaches.	SAML, UTSPH-EP	6/13	9/14
3.2	Develop a targeted list of needed species/watersheds for fecal sample collection and plan for their collection and delivery.	TWRI, IRNR, UTSPH-EP, SAML	2/13	6/13
3.3	IRNR and project partners will collect 100 fecal samples in accordance with the plan developed in Subtask 3.2	IRNR	7/13	5/15
4.1	Continued operation and maintenance of BST analytical equipment and support of personnel needs to sustain operating capability and expand the utilization of BST applications statewide.	SAML, UTSPH-EP	10/12	5/15
4.2 4.3	Maintain needed level of training of SAML and UTSPH-EP personnel.	SAML, UTSPH-EP	2/13	5/15
4.4	Expansion of Texas *E. coli* BST Library through analysis of approximately 100 known-source fecal samples collected by IRNR (see 3.3)	UTSPH-EP, SAML, IRNR	7/13	5/15
4.4	Data analysis for approximately 100 known-source *E. coli* isolates from the Leona River (TSSWCB project 11-50) for expansion of the Texas *E. coli* BST Library	UTSPH-EP, SAML	7/13	5/15
4.4	ERIC-RP Fingerprint & analyze 20 known source *E. coli* isolates from project 11-51(birds & bats)	SAML	7/13	5/15
4.5	Evaluation of geographical and temporal stability of the Texas *E. coli* BST Library and diversity of source-specific isolates	UTSPH-EP, SAML	7/13	5/15
4.6	Development/ evaluation of new source-specific bacterial markers (e.g., poultry, feral hog from domestic swine, deer from other ruminants) for library-independent BST	UTSPH-EP, SAML	7/13	5/15
5	Maintain project website; meet with natural resource agencies and researchers engaged in BST; distribute educational brochures; participate in conferences, workshops, etc.	TWRI, UTSHP-EP, SAML	2/13	5/15

Section A7: Quality Objectives and Criteria for Data Quality

The objective of this section is to ensure that data collected meets the data quality objectives (DQOs) of the project. The current project has a significant focus on the continued development and evaluation of qualitative BST analytical procedures. Project objectives include the continued development, updating and implementation of statewide BST template-SOPs for ERIC-PCR, RiboPrinting, and *Bacteroidales* PCR to standardize methodologies employed; continued development of the Texas *E. coli* BST Library by incorporation of additional known source fecal sample isolates, and; further evaluation of source-specific bacterial markers for library independent BST. Sampling to be conducted in conjunction with this project is primarily associated with expanding the Texas *E. coli* BST Library so it better represents the diversity and abundance of potential fecal sources that may contaminate surface water in watersheds throughout the State. The primary sampling design consideration is to fill gaps in the Texas *E. coli* BST Library identified in other TSSWCB-funded BST projects. The measurement performance criteria and data quality objectives for the BST analytical procedures are specified in Table A7.1.

Precision

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. For quantitative microbiological analyses, the method to be used for calculating precision is the one outlined in *Standard Methods for the Examination of Water and Wastewater*, 21st Edition, section 9020 B.8.b.

$$RPD_{bacteria} = (\log X_1 - \log X_2)$$

Relative percent deviation (RPD) $_{bacteria}$ should be lower than 3.27 ΣRlog/n, where Rlog is the difference in the natural log of duplicates for the first 15 positive samples. RiboPrinting and ERIC-PCR BST are qualitative assays, generating two different types of DNA fingerprints. The precision of the ERIC-PCR and RiboPrinting can be measured as the percent of *E. coli* isolates that, when typed multiple times, produce the same ultimate source result in terms of the source identified. Precision for ERIC-PCR and RiboPrinting will be determined using a laboratory control strain of *E. coli* (QC101). For ERIC-PCR and RiboPrinting, the data quality objective is 90% precision. *Bacteroidales* PCR, *B. avium* PCR and *E. coli* library-independent PCR based on ERIC-PCR DNA sequences are also currently presence-absence qualitative assays. Precision is determined using fecal DNA or *E. coli* isolate DNA from known human and animal sources with a data quality objective of 100% agreement in marker detection among replicates.

Representativeness

One of the goals of this project is to expand the Texas *E. coli* BST Library so it is more representative of the *E. coli* isolates found in known source samples throughout Texas watersheds. The ability to reach this goal is tempered by the availability of time and funding. To maximize resources, only one fecal sample per animal will be collected. Samples will be collected from animals in different locations throughout the watershed and state. In addition, for ERIC-RP, ERIC-PCR is first used to identify clonal (identical) *E. coli* isolates from each fecal sample to ensure appropriate representation of isolates selected for the library.

Table A7.1 Data Quality Objectives for Measurement Data

Parameter	Units	Method Type	Method	Method Description	Parameter Code	AWRL[1]	Precision of Laboratory Duplicates	Accuracy[2]	Percent Complete[3]
E. coli RiboPrint fingerprint	NA	DNA/ image matching	UTSPH-EP SOP	RiboPrinting	NA	NA	90% identical[4]	90% correct[4]	90
E. coli ERIC-PCR fingerprint	NA	DNA/ image matching	UTSPH-EP SOP	ERIC-PCR	NA	NA	90% identical[4]	90% correct[4]	90
Bacteroidales PCR	Qualitative marker	PCR presence or absence	UTSPH-EP SOP	*Bacteroidales* PCR	NA	NA	100% agreement for presence/ absence detection of markers	90% correct (presence/ absence)	90
E. coli library-independent PCR markers based on ERIC-PCR DNA sequences	Qualitative marker	PCR presence or absence	UTSPH-EP SOP	*E. coli* PCR	NA	NA	100% agreement for presence/ absence detection of markers	90% correct (presence/ absence)	90
B. avium PCR (Poultry marker)	Qualitative marker	PCR presence or absence	SAML SOP	*B. avium* PCR	NA	NA	100% agreement for presence/ absence detection of markers	90% correct (presence/ absence)	90

[1] minimum detection limits for field parameters represent manufacturer specifications and will be used for the AWRL in this instance.
[2] Manufacturer specifications are presented for accuracy limits and method detection limits for field parameters.
[3] The objective is for 90% of the data to be collected.
[4] Accuracy and laboratory method precision for BST will be determined using an *E. coli* QC isolate and DNA from known-source samples

Accuracy

Accuracy is a statistical measurement of correctness and includes components of systemic error. A measurement is considered accurate when the result reported does not differ from the true situation. Performance limits for all measured parameters are specified in Table A7.1.

Accuracy for BST methods will be assessed using the *E. coli* (QC101) lab control strain for ERIC-RP and fecal DNA or *E. coli* isolate DNA from known human and animal sources for *Bacteroidales* PCR, *B. avium* PCR, and *E. coli* library-independent PCR. For the ERIC-RP, the data quality objective is 90% accuracy for correct identification to library strain. For *Bacteroidales* PCR, *B. avium* PCR, and *E. coli* library-independent PCR the data quality objective is 90% accuracy for the presence/absence of the appropriate markers in control DNA samples. An additional element of accuracy is the absence of contamination. This is determined through the analysis of no template controls for the PCR methods.

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, library, 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 to the total potential data. Ideally, 100% of the data should 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 percent data completion is achieved.

Section A8: Special Training Requirements/Certifications

All personnel involved in sampling, sample analyses, and statistical analyses have received the appropriate education and training required to adequately perform their duties. No special certifications are required. Personnel involved in this project have been trained in the appropriate use of field equipment, laboratory equipment, laboratory safety, cryogenics safety, and all applicable SOPs. One of the objectives of this project is the continued support, training, and retention of skilled personnel. To meet the needs of the State, BST analytical capabilities will be maintained at both UTSPH-EP and SAML BST laboratories. Training needs for each individual laboratory's personnel will be coordinated to ensure appropriate technology transfer and comparability of BST data.

Section A9: Documentation and Records

Hard copies of general maintenance records, all field data sheets, COC forms, laboratory data entry sheets, calibration logs, and corrective action reports (CARs) will be archived by each laboratory as outlined in Table A9.1. In addition, UTSPH-EP and SAML will archive electronic forms of all project data for at least five years. 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 form is presented in Appendix A and a blank Chain of Custody (COC) record and Sheets of Lading for Fecal Specimen Transport are presented in Appendix B.

Quarterly Progress Reports (QPRs) will note 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.

Table A9.1 Project Documents and Records

Document/Record	Location	Retention	Form
QAPP, amendments, and appendices	TWRI	5 years	Paper/Electronic
Chain of custody records	SAML/UTSPH	2 years	Paper
Sheets of Lading for Fecal Specimens	SAML/UTSPH	2 years	Paper
Corrective action reports	TWRI	2 years	Paper/Electronic
Field notes	IRNR	2 years	Paper
Bacteriological data sheet	SAML/UTSPH	2 years	Paper
Laboratory QA manuals and/or SOPs	SAML/UTSPH	5 years	Paper/Electronic
Lab equipment calibration records & maintenance logs	SAML/UTSPH	2 years	Paper
Lab data reports/results	SAML/UTSPH	5 years	Paper/Electronic
Quarterly progress reports/final report/data	TWRI	5 years	Paper/Electronic

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 annually on the anniversary date, or revised and reissued within 120 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 plan is current. This will be accomplished by submitting a cover letter station 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 non-conformances; improve operational efficiency; and/or accommodate unique or unanticipated circumstances; Written requests for amendments are directed from the TWRI Project Leader or designee 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 Project Leader or designee. Amendments shall be reviewed, approved, and incorporated into a revised QAPP during the annual revision process.

Section B1: Sampling Process Design (Experimental Design)

Sampling to be conducted in conjunction with this project is primarily associated with expanding the Texas *E. coli* BST Library so it better represents the diversity and abundance of potential fecal sources that may contaminate surface water in watersheds throughout the State. The primary sampling design consideration is to fill gaps in the Texas *E. coli* BST Library identified in other TSSWCB-funded BST projects. Targeted species will include small mammals such as mice, squirrels, nutria and rabbits. In addition, samples will be collected from at least one previously studied watershed (e.g., Leon River) in order to determine the temporal stability of the Texas *E. coli* BST Library. Known-source fecal samples collected under this project will also be analyzed using for the human HF183 *Bacteroidales* marker to explore issues of cross-reactivity and false positives.

Approximately 100 known-source samples from different individual animals will be collected throughout the course of the project and will include domestic animals, wildlife and human sources. These known sources of bacteria (domestic animals, wildlife and humans) will not be collected from the same locations during every collection due to the nature of the animals. Sewage and septage samples will represent human sources (not individuals) and will be collected based on granted access to facilities.

Section B2: Sampling Method Requirements

Fecal Sampling Method Requirements

Fecal sample collections are described in Appendix C-1. To ensure fresh samples of known origin, fecal samples will be obtained using one of five methods: a) collected from animals visually observed defecating by technician; b) collected from cages of trapped animals; c) collected from intestines of animals recently killed by cars (within 24 hours); d) collected from intestines of animals legally harvested; or e) human (wastewater) samples collected from individual septic tanks, composite septic samples from pump trucks, wastewater treatment plant influent (for plants with secondary disinfection or lagoon treatment), or from lagoon treatment effluents. All fecal samples will be shipped to UTSPH-EP or SAML for BST analysis within 3 days of collection.

If trapping is required, project personnel will randomly locate trap arrays on properties where permission has been received in order to capture species that contribute to the bacteria impairment. Animals will be released safely and once clear of the area, technicians will collect feces. After releasing animals from the trap and collecting fecal samples, the cage will be cleaned and moved to prevent possible cross contamination of subsequent fecal samples. Traps will be closed every morning and reopened every evening during each trap session to prevent animals from being confined in cages in daylight hours. Traps will be set in shaded areas to reduce heat stress on the animals and for their safety. During periods of high temperature, trapping may be rescheduled.

Documentation of Field Sampling Activities

Recording Data

For the purposes of this section and subsequent sections, all field and laboratory personnel follow the basic rules for recording information as documented below:

- Legible writing with no modifications, write-overs or cross-outs;
- Correction of errors with a single line followed by an initial and date;
- Close-outs on incomplete pages with an initialed and dated diagonal line.

Each fecal sample will be collected aseptically in a new, sterile fecal tube (Sarstedt, cat# 80.734.311). Wastewater samples can initially be collected with sterile bottles or other suitable device and then transferred to the fecal tubes. Specimen tubes will be labeled with:

a. Sampling date
b. Sampling time
c. Animal species
d. Sample location (e.g., GPS coordinates [preferred] or town, city, and/or county)
e. Sample collector’s name, initials
f. Any other pertinent information, e.g., sex of animal or any other easily obtainable information such as beef cattle versus dairy cattle

All the sample information will be logged into a field log. Samples should be refrigerated (~4°C) or kept on ice following collection and shipped to the designated laboratory on ice within 3 days of collection. See SOP in Appendix C for complete protocol.

Safety is an issue when working with fecal samples due to the bacterial concentration. Hazardous material safety handling instructions will be included in a file for driver to carry that will be visible on seat or dash of vehicle in case of accident or being stopped by law enforcement officers. Biohazard signs will be placed on the cooler containing samples collected for transport. Sheets of Lading (Appendix B) will be on hand with the field technician and completed for each fecal sample collected along with a COC form.

Table B2.1 Sample Volume, Container Types, Minimum Sample Volume, Preservation Requirements, and Holding Time Requirements.

Parameter	Matrix	Container	Preservation	Temperature	Sample Volume	Holding Time
E. coli	Feces, sewage	Sterile screw-capped polypropylene tube	none	4°C	>1 mg	3 days

Section B3: Sample Handling and Custody Requirements

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 and inter-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 will sign the COC and transport it with the sample to the laboratory. 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. Sample numbers will then be recorded into a laboratory sample log, where the laboratory staff member who receives the sample will sign it. A copy of a blank COC form used on this project is included as Appendix B.

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 Supervisor has the responsibility to ensure that holding times are met with fecal 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 are to be immediately reported to the TSSWCB PM. Failures 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 Project Leader and the TSSWCB PM/QAO will determine if the procedural violation may have compromised the validity of the resulting data. Any failure that potentially compromises data validity will invalidate data, and the sampling event should be repeated. CARs will be reported to the TSSWCB in the QPR. The CARs will be maintained by the TWRI Project Lead.

Section B4: Analytical Method Requirements

BST Analysis

The analytical methods utilized in BST analysis and sample preparation are listed in Table B4.1 and Table A7.1 and described in detail in Appendix C. 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.

Fecal specimens or domestic sewage samples collected by IRNR and project partners and sent to UTSPH-EP and SAML will be streaked (resuspended in buffer if necessary) onto modified mTEC medium. Modified mTEC medium is used in USEPA Method 1603 for water samples. Its use for source samples helps avoid selection of different types of *E. coli* due to different media. 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. Inoculated plates will be incubated at 35±0.5^{o}C for 2 hours to resuscitate stressed bacteria and then incubated at 44.5±0.2^{o}C for approximately 20 to 24 hours.

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 for the source sample isolates. Cultures of selected isolates will be archived using glycerol freezing medium (-80^{o}C). Up to 5 isolates per sample will be archived for fecal samples.

Three confirmed *E. coli* bacterial colonies from each source sample will be screened for clones (identical strains from the same sample) using a repetitive sequence polymerase chain reaction (rep-PCR) method. 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. 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 QA/QC, standardized protocols for PCR and image processing, and adequate training of personnel is crucial for generation of comparable data.

Non-clonal isolates will then be RiboPrinted. 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').

After screening the ERIC-RP fingerprints of these known source samples for host specificity, they will be included in the Texas *E. coli* BST Library of *E. coli* isolates from known animal and human sources collected throughout Texas.

Library-Independent Methods Development

Using known source fecal material, SAML and UTSPH-EP will use the best available bacterial indicators to evaluate and further develop/refine source-specific bacterial PCR markers. Specifically, efforts will be made on markers to 1) identify poultry litter/manure pollution, 2) evaluate the use of genetic targets based on ERIC-PCR products to differentiate human and animal derived *E. coli*, 3) differentiate between domestic swine and feral hogs using *Bacteroidales* PCR, 4) differentiate deer from other ruminants by continued analysis of existing data on deer fecal microbial communities, and 5) evaluate occurrences of *Bacteroidales* human HF183 marker cross reactivity for all 100 known source animal samples collected in this study.

Failures in Measurement Systems and Corrective Actions

Failures in 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 UTSPH-EP 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 QAO will include this information in the CAR and submit with the QPR which is sent to the TSSWCB PM.

Table B4.1 Laboratory Analytical Methods

Laboratory Parameter	Method	Equipment Used
E. coli ERIC-PCR fingerprint	UTSPH-EP SOP	PCR thermal cycler, gel electrophoresis app
E. coli RiboPrint fingerprint	UTSPH-EP SOP	RiboPrinter
Bacteroidales PCR	UTSPH-EP SOP	PCR thermal cycler, gel electrophoresis app
B. avium PCR	SAML SOP	PCR thermal cycler, gel electrophoresis app

SOP = Standard Operating Procedure

Section B5: Quality Control Requirements

Laboratory Measurement Quality Control Requirements and Acceptability Criteria

Method Specific QC requirements
QC samples, other than those specified later this section, are run (e.g., sample duplicates, surrogates, internal standards, continuing calibration samples, interference check samples, positive control, negative control, and media blank) as specified in the methods. The requirements for these samples, their acceptance criteria or instructions for establishing criteria, and corrective actions are method-specific.

Laboratory Duplicates
A laboratory duplicate is prepared by taking aliquots of a sample from the same container under laboratory conditions and processed and analyzed independently. A laboratory control sample duplicate (LCSD) is prepared in the laboratory by splitting aliquots of an LCS. Both samples are carried through the entire preparation and analytical process. LCSDs are used to assess precision and are performed at a rate of one per preparation batch. Measurement performance specifications are used to determine the acceptability of duplicate analyses as specified in Table A7.1.

Method blank
A method blank is a sample of matrix similar to the batch of associated samples (when available) that is free from the analytes of interest and is processed simultaneously with and under the same conditions as the samples through all steps of the analytical procedures, and in which no target analytes or interferences are present at concentrations that impact the analytical results for sample analyses. The method blanks are performed at a rate of once per preparation batch. The method blank is used to document contamination from the analytical process. For each of the analytical methods used in this project, method blanks should test negative for the target analytes/markers. In addition, no template negative controls will be analyzed for each batch of PCR. Samples associated with a contaminated blank shall be evaluated as to the best corrective action for the samples (e.g. reprocessing or data qualifying codes). In all cases the corrective action must be documented.

Table A7.1 lists the required accuracy, precision, and completeness limits for the parameters of interest. It is the responsibility of the Project Leader to verify that the data are representative. The 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 quality assurance of field sampling methods will be conducted by the TSSWCB QAO or their designee at least once per the life of the project.

Positive Controls

Positive controls will consist of a laboratory control strain of *E. coli* (QC101) for ERIC-PCR and RiboPrinting and will be included in every preparation batch. For PCR-based library independent BST methods positive controls will consist of fecal DNA or *E. coli* isolate DNA from known human and animal sources and will be included with every preparation batch. Positive controls should always test positive. Samples associated with a failed positive control shall be evaluated as to the best corrective action for the samples (e.g. reprocessing or data qualifying codes). In all cases the corrective action must be documented. The analytical methods are listed in Table A7.1 of Section A7. No EPA-approved methods exist for BST. Detailed SOPs for these methods are provided in Appendix C.

Failures in Quality Control and Corrective Action

Notations of blank contamination will be noted in the QPR. 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. CARs will be maintained by the TWRI Project Lead.

Section B6: Equipment Testing, Inspection, & Maintenance Requirements

To minimize downtime of all measurement systems, spare parts for laboratory equipment (Table B6.1) will be kept in the laboratory (when feasible), and all laboratory equipment will be maintained in 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, 22nd Edition. Maintenance and inspection logs will be kept on each piece of laboratory equipment. 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. CARs will be maintained by the TWRI Project Lead.

Table B6.1 Equipment Inspection and Maintenance Requirements

Equipment	Relevant Testing, Inspection & Maintenance Requirements
Thermometers	SM 9020 B 3.a
PCR Thermal cycler	Per manufacturer
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 3.a
Utensils and containers	SM 9020 B 3.b
Dilution water bottles	SM 9020 B 3.c

Section B7: Instrument Calibration and Frequency

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 USEPA-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 either a laboratory or field audit.

All instruments or devices used in obtaining environmental data will be used according to appropriate laboratory or field 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 National Institute of Standards and Technology (NIST) traceable whenever possible. When NIST traceability is not available, standards shall be of American Chemical Society 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. CARs will be maintained by the TWRI Project Lead.

Table B7.1 Instrument Calibration Requirements

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

Section B8: Inspection/Acceptance Requirements for 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 quality control 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.

Section B9: Data Acquisition Requirements (Non-direct Measurements)

All required data to be used for this project will be collected in accordance with this QAPP.

BST Analysis

Data analyzed using BST analysis methods for this project will consist of data produced during the course of this study under the specifics of this QAPP, or generated under previous TSSWCB studies with accepted QAPPs.

Section B10: Data Management

Laboratory Data

All field samples (known-source fecal samples) will be logged upon receipt, COC forms (if applicable) will be checked for number of samples, proper and exact identification number, signatures, dates, and type of analysis specified. TSSWCB will be notified if any discrepancy is found and laboratory analysis will not occur until proper corrections are made. All samples will be stored at 4ºC until analysis. Bacteriological samples will be given a unique identification number and logged into a Microsoft Excel database used to store field data. All backup and safety features of this database are the same as explained above. Enumerated bacteriological data will be manually entered into the database system for electronic storage. At least 10% of all data manually entered in the database will be reviewed for accuracy by the Project Lead to ensure that there are no transcription errors. Hard copies of data will be printed and housed at the generating laboratory for a period of five years. Any COC's and bacteriological records related to QA/QC of bacteriological procedures will be housed at UTSPH-EP and SAML.

Sample Delivery to Other Laboratories

Fecal samples for BST analysis will be collected and logged using the procedures described above in the field collection and lab data sections. The Research Technician ensures that these samples are handled according to procedures laid out in this QAPP and that COC forms are correctly filled out for sample delivery to the UTSPH-EP and SAML. The Research Technician ships the samples, the appropriate Sheets of Lading for Fecal Specimen Transport (Appendix B) and COC forms to the UTSPH-EP and SAML labs via FedEx in an appropriately labeled container that maintains appropriate sample temperatures with the use of dry ice. Once the samples are received at the lab, the COC forms are updated and the Research Technician is notified of the samples receipt.

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 UTSPH-EP Project Co-Lead or SAML Project Co-Lead, 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 Reporting

Data will be reported according to the standards of the TSSWCB. A data review checklist (Appendix D) will assist in ensuring that the reported data are reported correctly.

Data Dissemination

At the project's conclusion, the TWRI Project Lead will provide a copy of the complete project electronic database via recordable CD-ROM media to the TSSWCB PM, along with the final report. TSSWCB may elect to take possession of all project records or records will be maintained according to the Project Records retention schedule in Table A.9. Summaries of the data will be presented in the final project report. TSSWCB may disseminate validated data and reports.

Section C1: Assessments and Response Actions

Table C1.1 presents the types of assessments and response action for activities applicable to this QAPP.

Table C1.1. Assessments and Response Actions

Assessment Activity	Approximate Schedule	Responsible Party	Scope	Response Requirements
Status Monitoring Oversight, etc.	Continuous	TWRI	Monitor project status and records to ensure requirements are being fulfilled. Monitoring & review performance & data quality	Report to TSSWCB in QPR.
Equipment testing	As needed	SAML, UTSPH-EP	Pass/Fail equipment testing	Repair or replace
Data completeness	As needed	SAML, UTSPH-EP	Assess samples analyzed vs. planned analysis	Reanalyze or amend objectives
Laboratory Inspections	TBD by TSSWCB	TSSWCB	Analytical and QC procedures in the laboratory	30 days to respond to TSSWCB with corrective actions
Technical systems audit	As needed	TSSWCB	Assess compliance with QAPP; review facility and data management as they relate to the project	30 days to respond to TSSWCB with corrective actions
Monitoring Systems Audit	Once per life of project	TSSWCB	Assess compliance with QAPP; review field sampling and data management as they relate to the project	30 days to respond to TSSWCB with corrective actions

Corrective Action

The Project Leaders are responsible for implementing and tracking corrective action procedures as a result of audit findings. Records of audit findings and corrective actions are maintained by the TSSWCB QAO.

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.

Section C2: Reports to Management

QPRs will be generated by TWRI and will note activities conducted in connection with the water quality monitoring program, 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. 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.

TWRI will work with AgriLife SCSC and UTSPH EP to develop a Final Report for submission to the TSSWCB that summarizes activities completed, conclusions reached during the project, and the extent to which project goals and measures of success have been achieved.

Section D1: Data Review, Validation, and Verification

All data obtained from field and laboratory measurements will be reviewed and verified for integrity, continuity, reasonableness, and conformance to project requirements, and then validated against the DQOs outlined in Section A7. Only those data that are supported by appropriate QC data and meet the DQOs defined for this project will be considered acceptable for use.

The procedures for verification and validation of data are described in Section D2, below. Project Leaders are responsible for ensuring that field and laboratory data collected are properly reviewed, verified, and submitted in the required format for the project database. TWRI is responsible for validating that all data collected meet the DQOs of the project are suitable for submission to TSSWCB.

Section D2: Validation and Verification Methods

All data will be verified to ensure they are representative of the samples analyzed and locations where measurements were made, and that the data and associated QC data conform to project specifications. The TWRI Project Lead is responsible for the integrity, validation, and verification of the data each field and laboratory task generates or handles throughout each process. The field and laboratory QA tasks ensure the verification of field 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 Section B5) and are checked to ensure the verification of raw data for errors, especially errors in transcription, calculations, and data input. Potential outliers are identified by examination for unreasonable data, or identified using computer-based statistical software. 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 that 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 TSSWCB QAO to establish the appropriate course of action, or the data associated with the issue are rejected. Performance of these tasks is documented by completion of the data review checklist (Appendix D).

Project Leaders and TWRI are responsible for validating that the verified data are scientifically sound, defensible, of known precision, accuracy, integrity, meet the DQOs of the project, and are reportable to the TSSWCB.

Table D2.1 Data Review, Verification, and Validation Procedures

Data to be Verified	Field† Supervisor	Laboratory Supervisor	PM/QAO Task‡
Collection & analysis techniques consistent with SOPs & QAPP	X	X	X
Field QC samples collected for all parameters as prescribed in the QAPP	X		X
Field documentation complete	X		X
Instrument calibration data complete	X	X	X
Sample documentation complete	X	X	X
Field QC results within acceptance limits	X		X
Sample identifications	X	X	X
Chain of custody complete/acceptable	X	X	X
Sample preservation and handling	X	X	X
Holding times	X	X	X
Instrument calibration data	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		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

† Field and Laboratory Supervisor may be the same person

‡ TSSWCB PM / QAO will monitor data for QA/QC purposes as needed.

All other entities are required to inspect 100% of the data prior to approval

Section 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. Corrective Action Reports 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 data quality objectives 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 data quality objectives described in this QAPP will be reported and included in the final project report. Since BST is an evolving science and no EPA-approved protocols currently exist, a discussion of the uncertainties surrounding source identification and the appropriate use of BST results will be included in the project final report. Data and information produced thru this project will provide needed information pertaining to Texas BST efforts.

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APPENDIX A

Corrective Action Report

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:_____

APPENDIX B

Chain of Custody Record
&
Sheets of Lading for Fecal Specimen Transport Template

CHAIN OF CUSTODY RECORD

Project:	**Remarks:**
Name and signature of collector:	**Air bill #**

Station ID	**Sample ID**	**Media Code**	**Sample Type**	**Preservative**	**Collection Date**	**Time**

Relinquished by:	Date:	Time:	Received by:	Date:	Time:
Laboratory Notes:					
Media Code: (**FS**) Fecal Sample; (**SS**) Sewage Sample					

Sheets of Lading for Fecal Specimen Transport

(Collector's Organization)

FY13-14 Statewide BST Program

(Collector's Name and title)
(Collector's Phone Number)

In case of EMERGENCY:
(Contact name and number)

Date:_____Time:_____

Sample: Fecal **Hazard:** Bacteria

Species/ Animal:_____

Photo: Yes No

GPS (or other location note): Lat_____Long_____

_____

Other Info:_____

Technician:_____

APPENDIX C

BST STANDARD OPERATING PROCEDURES

C-1: Collection of Fecal Samples for Bacterial Source Tracking

1. Only fresh fecal samples of known origin should be collected. Specifically, fecal samples should be obtained in one of five ways:
   a. Collected from animals visually observed defecating by technician.
   b. Collected from trapped animals.
   c. Collected from intestines of animals legally harvested.
   d. Collected from the intestines of animals recently killed by cars (within 24 hours).
   e. Human (wastewater) samples collected from individual septic tanks, composite septic samples from pump trucks, from wastewater treatment plant influent (for plants with secondary disinfection or lagoon treatment), or from lagoon treatment effluents.

2. Samples should be carefully collected to avoid contamination. Samples on the ground should be collected with a sterile spatula, or similar device, while avoiding collection of material in contact with soil or other possible sources of contamination. Intestinal samples should be collected from animals by using sterile loops inserted anally or by cutting into the intestine using a sterile scalpel. Wastewater samples can initially be collected with sterile bottles, or other suitable device and then transferred to the fecal tubes described below.

3. Each fecal sample should be placed in a new, sterile fecal tube (Sarstedt, cat# 80.734.311). Tubes should be filled approximately ¾ full (can provide less material for smaller animals)

4. Samples should be refrigerated (~4°C) or kept on ice following collection.

5. At the time of sampling, record detailed information on the tube regarding the sample including:
   a. Sampling date
   b. Sampling time
   c. Animal species
   d. Sample location (e.g., GPS coordinates [preferred] or town, city, and/or county)
   e. Sample collector's name/initials
   f. Any other pertinent information, e.g. sex of animal or any other easily obtainable information such as beef cattle versus dairy cattle

6. Notify the appropriate lab via email or phone as soon as possible (prior to or immediately following sample collection) with an estimated number of samples that will be shipped and the expected date of shipment. This will allow lab to make appropriate preparations to process the samples immediately upon arrival.

SAML
Emily Martin or Heidi Mjelde
emartin@ag.tamu.edu or
hmjelde@ag.tamu.edu
979-845-5604

UTSPH-EP
Elizabeth Casarez or George Di Giovanni
elizabeth.a.casarez@uth.tmc.edu or
george.d.digiovanni@uth.tmc.edu
915 747-8076 or 915 747-8509

7. Samples should be shipped (at 4ºC) as soon as possible (within **3 days**) to the appropriate lab (address below). 'Blue-ice' or freezer blocks should be used to keep the samples cool, but not frozen during transport. Samples should be placed in secondary containment such as large Whirl-Pak or zip-top bags.

8. Notification of shipment should be sent to the appropriate lab via email or phone (see contact info above) no later than the day of overnight shipping. Notification should include tracking number and direct collections contact person for confirmation upon receipt of samples.

9. Ship samples (and COCs) in insulated coolers (marked on outside to indicate that contents are perishable) with sufficient ice packs to maintain ~4°C to:

SAML
Terry Gentry
Texas A&M University
Soil & Crop Sciences; Heep Center 539
370 Olsen Blvd
College Station, TX 77843
979-845-5604

UTSPH-EP
George Di Giovanni
UT-Houston School of Public Health
Biology Building B224
500 W. University
El Paso, TX 79968
915-747-8509

C-2: Laboratory Protocol for Isolation and Confirmation of *Escherichia coli* from Fecal Specimens

Note: All collection and handling of fecal specimens should be performed using protective gear (e.g. latex or nitrile gloves). Specimens should be handled aseptically to ensure sample quality and minimize exposure of personnel to pathogens. All feces collected will be placed in screw capped sterile containers. Containers will be labeled with: Name of collector, date, species, GPS location, and photo of specimen before collection. Containers will then be placed in ziplock biohazard bags with lading pouch. Information will be written on lading report and placed in the bag. Fecal specimens will be placed in an insulated cooler on ice during transport.

Note: All handling of fecal specimens and cultures will be performed using a Class 2 biological safety cabinet to minimize the exposure of laboratory personnel to pathogens.

1. Using a bacteriological loop, streak a loop full of fecal material onto a labeled modified mTEC agar plate (USEPA-821-R-02-023, Modified USEPA Method 1603; http://www.epa.gov/nerlcwww/1603sp02.pdf) for isolation of *E. coli* colonies.

2. Incubate the plate inverted at 44.5±0.2°C for 20 to 24 h.

3. Examine the plate for presumptive *E. coli* colonies, which will appear red or magenta colored.

4. Select up to three presumptive *E. coli* colonies and streak each colony for purity onto a labeled nutrient agar MUG (NA-MUG) plate.

5. Invert and incubate plates at 35 to 37°C for 20 to 24 h.

6. Examine the cultures using a **long-wave handheld UV lamp**. If there is a mixture of fluorescent and non-fluorescent colonies, select a well isolated fluorescent colony and streak again onto NA-MUG for purity.

7. At the discretion of the laboratory, additional biochemical tests such as urease, indole, and citrate tests may be performed.

C-3: 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.

C-4: 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 in cold room (4°C) 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)

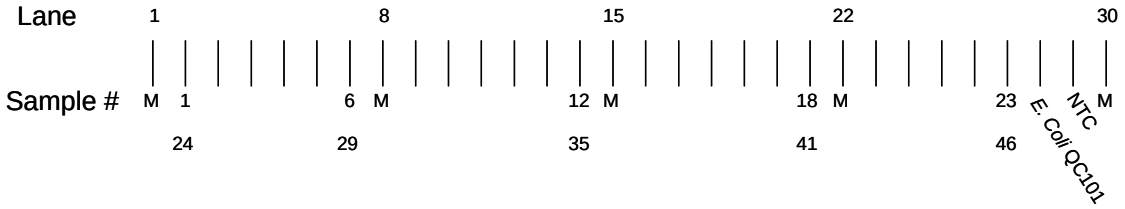
    f. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes

    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 Logic 200 SOP for image capture. Save digital photograph as a TIFF file (default) and print a hardcopy for notebook.

C-5: 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.

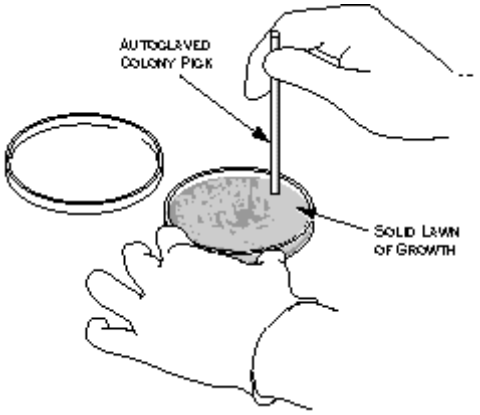
2. For Gram negative samples (including *E. coli*), add 200 µL of sample buffer from the intermediate tube.

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

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.

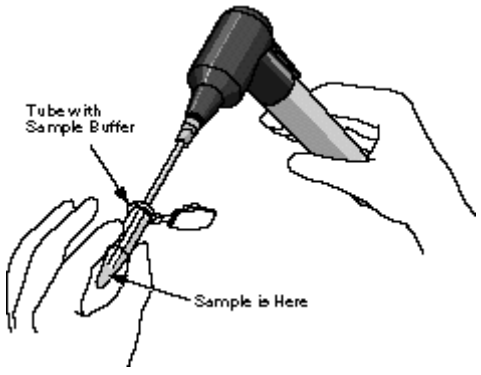
3. Press the ON lever on the mixer for about 5 seconds.

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

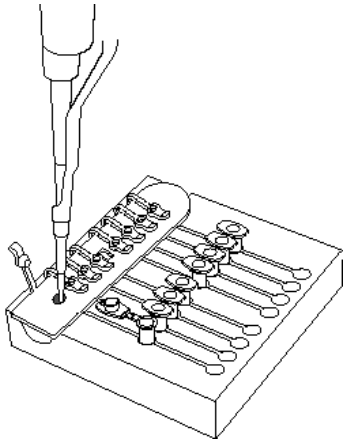
5. For **Gram positive samples only**, (e.g. *Staphylococcus* and *Listeria*) locate a new colony pick and repeat the steps for harvesting and mixing samples, adding a second sample to the original tube. Discard the used picks in a biowaste bag.

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.

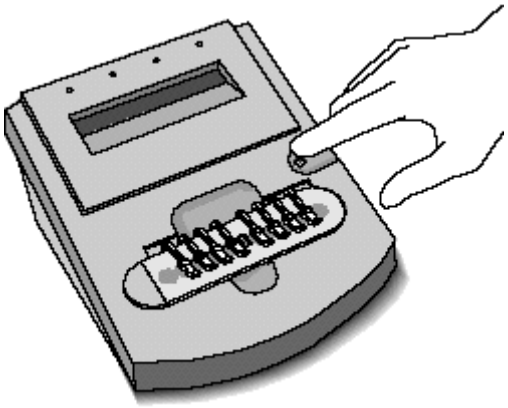
6. Lightly wipe down the outer surfaces of the sample carrier with a lab wipe wetted with surface disinfectant (10% bleach or 70% alcohol).

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, and 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.

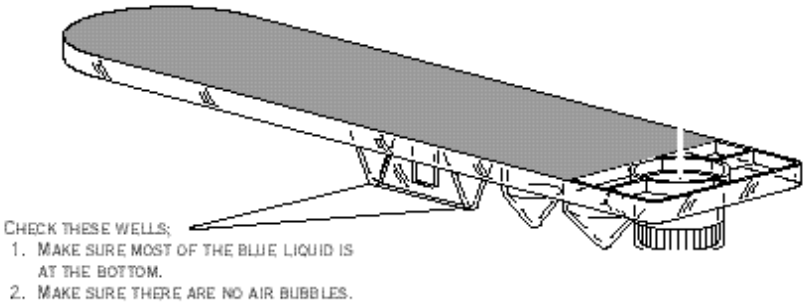
2. Check the wells in the carrier. If most of the liquid appears to be in the bottom of the wells and there are no bubbles, go to step 3. Otherwise **lightly tap the side of the carrier a few times with your finger to release any material that has adhered to the lid.**

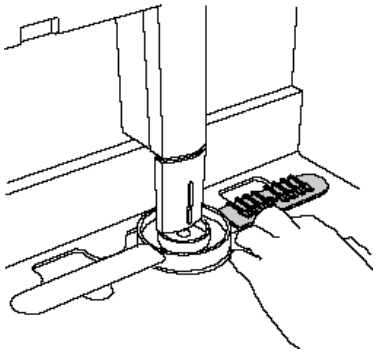
3. CAUTION! Do not tap the carrier briskly. This may cause the marker to degrade which can create inaccurate results.

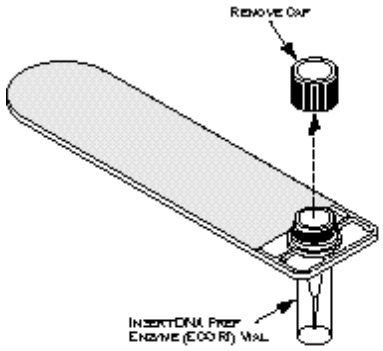
4. Remove a vial of DNA Prep Enzyme (*Hin*d III or *Eco*R I) from the freezer. ***Hin*d III (NEB Cat. #R0104M) is prepared in a Sarstedt 500-µL microfuge tube (Cat. #72730-005) as a 50 U/µL working stock as follows.**

   <u>50 U/µL:</u> 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.





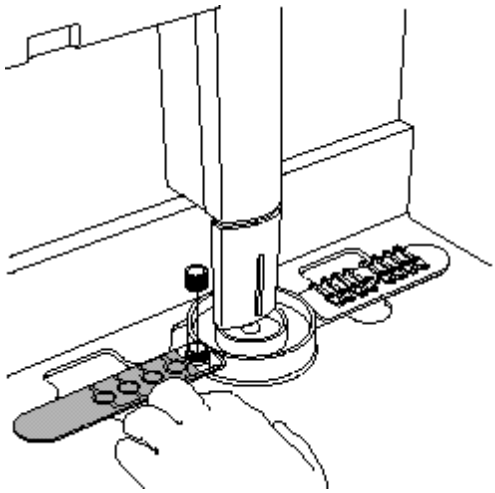


5. Remove the cap from the Enzyme vial.

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.

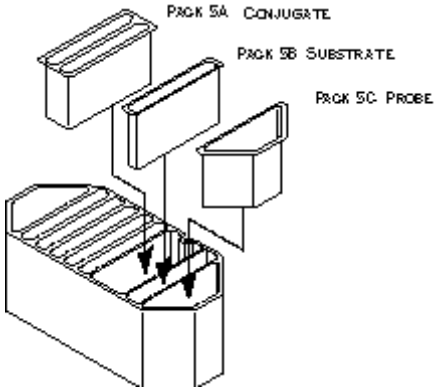


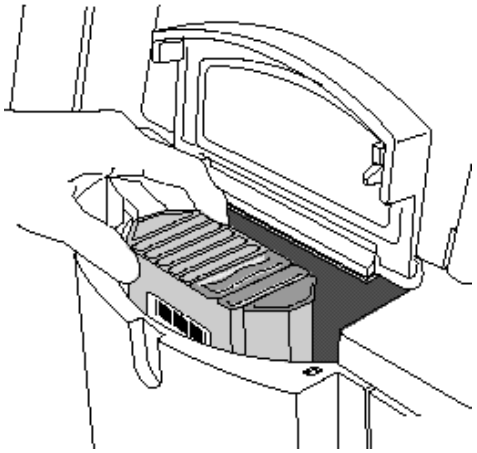
4. Load the MP Base and Carousel

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.

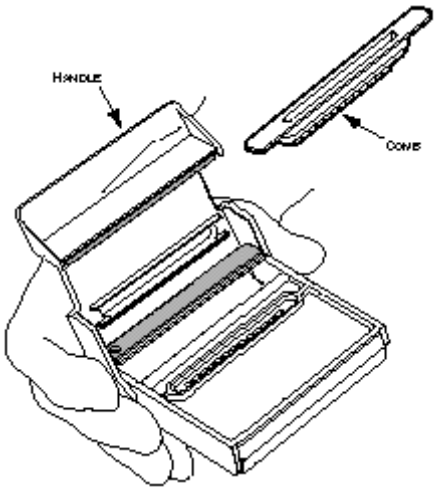


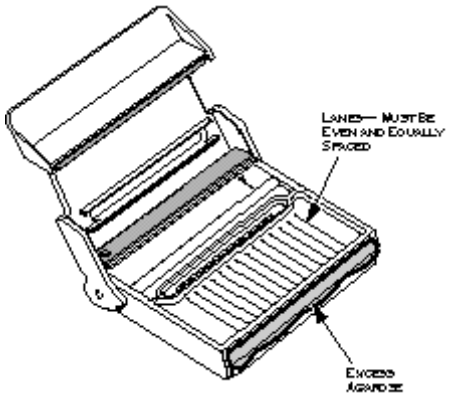


CAUTION! Push each insert firmly into place. If part of the insert extends above the top of the base, it could catch on the bottom of the deck and cause a system error. You could lose one or more batches as a result. Each insert is keyed by shape and cannot be inserted incorrectly.

5. Load the Gel Cassette

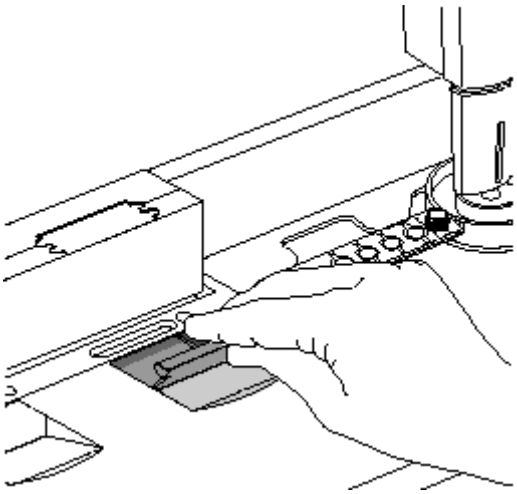
1. Remove the gel cassette from its package.
2. Grasp one end of the rubber comb and gently pull the comb from the cassette.
3. Unfold the handle of the cassette towards you until the handle snaps into place.
4. Check the front edge of the gel cassette and the lanes of the gel.





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.

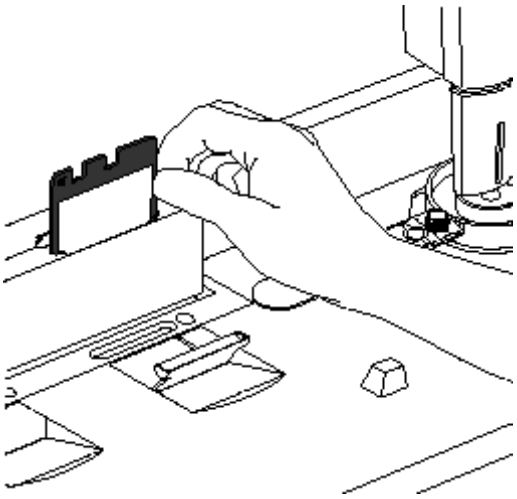


6. Press the cassette forward firmly until it snaps into place.

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

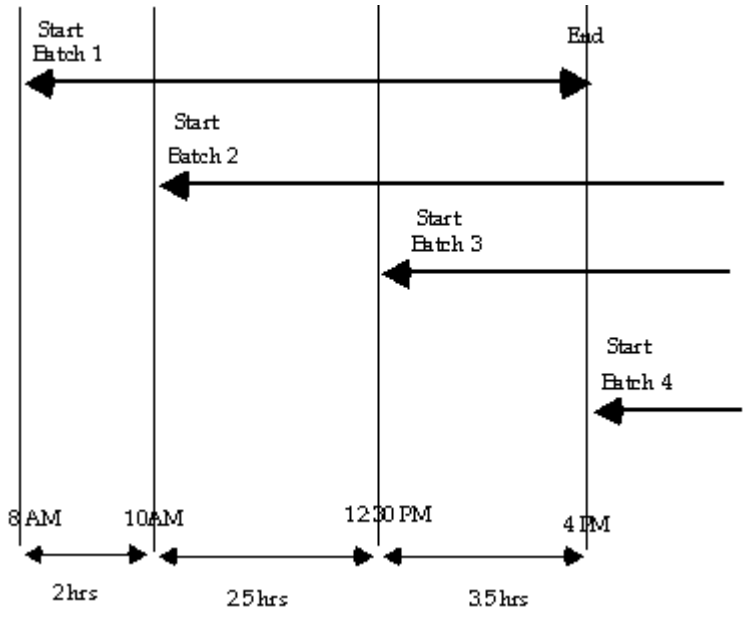
The RiboPrinter® microbial characterization system lets you load up to four VCA batches in an eight hour period. Other batches may take longer to process.
The chart above shows the approximate loading times for each batch in a work shift using only the VCA protocol.

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.



C-6: *Bacteroidales* PCR

DNA Extraction and PCR

1. DNA is extracted samples using a 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 (µL)	Final Calc	Final Units
Molecular Grade Water	**30.2**		
10X PCR buffer I w Mg (ABI)	**5**	1	X
$MgCl_2$ (25 mM) (ABI)	**1**	0.5 (2.0 final)	mM
each dGTP, dCTP, dATP (33 mM mix) (Amersham)	**0.3**	200	µM each
dUTP (100 mM) (Amersham)	**0.2**	400	µM
Bacteroidales Primer Mix	**5**	200	nM each
BSA (30 mg/mL)	**2.5**	1.5	µg/µL
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.

C-7: *B. avium* PCR

DNA Extraction and PCR

1. DNA is extracted samples using a 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.

B. avium 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.

B. avium **PCR Master Mix – per sample**

MASTER MIX	Amt (µL)	Final Calc	Final Units
Molecular Grade Water	**12.62**		
10X PCR buffer I w Mg (ABI)	**2.5**	1	X
$MgCl_2$ (25 mM) (ABI)	**0.5**	0.5 (2.0 final)	mM
each dGTP, dCTP, dATP (33 mM mix) (Amersham)	**0.15**	200	µM each
dUTP (100 mM) (Amersham)	**0.1**	400	µM
B. avium Primer Mix	**2.5**	200	nM each
BSA (30 mg/mL)	**1.25**	1.5	µg/µL
AmpliTaqGold (Units)	**0.25**	2.5	Units/rxn
Uracil DNA glycosylase NEB (UDG; 1 U/rxn)	**0.13**	0.5	Units/rxn

2. Dispense 20 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 *B. avium* program with the following cycling conditions:

    e. UDG digestion 50°C for 10 min

    f. Initial denaturation at 95°C for 15 min

    g. 45 Cycles:
        i. Denaturation at 95°C for 30 sec
        ii. Annealing at 60°C for 30 sec
        iii. Extension at 72°C for 30 sec

    h. 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.

APPENDIX D

Data Review Checklist
&
Data Summary Sheet

Data Review Checklist

Title of associated QAPP: _____

√, X, or N/A

Data Format and Structure

A. Are there any duplicate *Tag ID* numbers? _____
B. Are the *Tag prefixes* correct? _____
C. Are all *Tag ID* numbers 7 characters? _____
D. Are TCEQ station location (SLOC) numbers assigned? _____
E. Are sampling *Dates* in the correct format, MM/DD/YYYY? _____
F. Is the sampling *Time* based on the 24-hour clock (e.g. 13:04)? _____
G. Is the *Comment* field filled in where appropriate (e.g. unusual occurrence, sampling problems, unrepresentative of ambient water quality) and any punctuation deleted?

_____

H. *Source Code 1, 2* and *Program Code* are valid and used correctly? _____
I. Is the sampling date in the *Results* file the same as the one in the *Events* file? _____
J. Values represented by a valid parameter (*STORET*) code with the correct units and leading zeros? _____
K. Are there any duplicate parameter codes for the same *Tag Id*? _____
L. Are there any invalid symbols in the Greater Than/Less Than (*GT/LT*) field? _____
M. Are there any tag numbers in the *Results* file that are not in the *Events* file? _____
N. Have confirmed outliers been identified? (with a ■1" in the Verify_flg field) _____
O. Have grab data (bacteria, for example) taken during 24-hr events been reported separately as RT samples? _____
P. Is the file in the correct format (ASCII pipe-delimited text)? _____

Data Quality Review

A. Are all the values reported at or below the AWRL? _____
B. Have the outliers been verified? _____
C. Checks on correctness of analysis or data reasonableness performed?
e.g.: Is ortho-phosphorus less than total phosphorus?
Are dissolved metal concentrations less than or equal to total metals? _____
D. Have at least 10% of the data in the data set been reviewed against the field and laboratory data sheets? _____
E. Are all parameter codes in the data set listed in the QAPP? _____
F. Are all stations in the data set listed in the QAPP? _____

Documentation Review

A. Are blank results acceptable as specified in the QAPP? _____
B. Were control charts used to determine the acceptability of field duplicates? _____
C. Was documentation of any unusual occurrences that may affect water quality included in the Event file Comments field? _____
D. Were there any failures in sampling methods and/or deviations from sample design requirements that resulted in unreportable data? If yes, explain on next page. _____
E. Were there any failures in field and laboratory measurement systems that were not resolvable and resulted in unreportable data? If yes, explain on next page. _____

√ = Yes **X = No** **N/A** = Not applicable

Describe any data reporting inconsistencies with AWRL specifications. Explain failures in sampling methods and field and laboratory measurement systems that resulted in data that could not be reported to the TCEQ. (attach another page if necessary):

_____

_____

_____

_____

_____

Date Submitted to TCEQ: _____
Tag ID Series: _____
Date Range: _____
Data Source: _____
Comments (attach README.TXT file if applicable):

_____

_____

_____

_____

Planning Agency's Data Manager Signature: _____

Date: _____