Clean Water Act §319(h) Nonpoint Source Grant Program

Watershed Protection Plan Development for Buck Creek

TSSWCB Project # 06-11

Quality Assurance Project Plan

Texas State Soil and Water Conservation Board

Revision 2

prepared by

Texas AgriLife Research - Texas Water Resources Institute
Texas AgriLife Research and Extension Center at Vernon
Texas AgriLife Research and Extension Center at El Paso

Effective Period: October 1, 2006 to September 30, 2010

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Section A1: Approval Sheet

Watershed Protection Plan Development for Buck Creek

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Title: Associate Professor, Environmental Microbiology; Lab Manager; Project Co-Leader

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Texas AgriLife Research and Extension Center at Vernon (AgriLife Vernon)

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Title: RRA Laboratory Supervisor

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Name: W. Scott Burns
Title: RRA QAO

Signature: ______________________________ Date:____________________

Texas AgriLife Research - Texas Water Resources Institute (TWRI)

Name: B. L. Harris
Title: TWRI, Acting Director; Project Coordinator

Signature: ______________________________ Date:____________________

Name: Lucas Gregory
Title: TWRI QAO

Signature: ______________________________ Date:____________________

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List of Acronyms and Abbreviations

AgriLife El Paso	Texas AgriLife Research and Extension Center at El Paso
AgriLife Vernon	Texas AgriLife Research and Extension Center at Vernon
AWRL	Ambient Water Reporting Limit
BHI	Brain-Heart Infusion
BMPs	best management practices
BST	bacterial source tracking
CAR	corrective action report
CR	county road
CFU	colony forming units of bacteria
COC	chain of custody
CRP	Texas Clean Rivers Program
CWA	Clean Water Act
DO	dissolved oxygen
DNA	deoxyribonucleic acid
DQO	data quality objectives
E. coli	Escherichia coli
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction
FM	Farm to Market Road
GPS	global positioning system
MP	membrane and probe
mTEC	membrane Thermotolerant *E. coli*
MUG	4-methylumbelliferyl-β-D-glucuronide
NA-MUG	Nutrient Agar 4-methylumbelliferyl-β-D-glucuronide
NELAC	National Environmental Laboratory Accreditation Conference
NIST	National Institute of Standards and Technology
NPS	nonpoint source
PCR	polymerase chain reaction
PM	project manager
QA	quality assurance
QAO	quality assurance officer
QAPP	quality assurance project plan
QC	quality control
qPCR	quantitative PCR
QPR	quarterly progress report
RPD	relative percent deviation
RRA	Red River Authority of Texas
SH	state highway
SM	Standard Methods for the Examination of Water and Wastewater
SOP	Standard Operating Procedure
SWCD	Soil and Water Conservation District

TBE	Tris/Borate/EDTA Buffer
TCEQ	Texas Commission on Environmental Quality
TMDL	total maximum daily load
TSSWCB	Texas State Soil and Water Conservation Board
TWRI	Texas Water Resources Institute
USEPA	United States Environmental Protection Agency
USGS	United States Geological Survey
UV	ultraviolet light
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:

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Name: James Quashnock
Title: RRA Laboratory Manager

Name: W. Scott Burns
Title: RRA QAO

Section A4: Project/Task Organization

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

USEPA – Provides project oversight and funding at the federal level.

Henry Brewer, USEPA Texas Nonpoint Source Project Officer
Responsible for overall performance and direction of the project at the federal level. Ensures that the project assists in achieving the goals of the clean water act (CWA). Reviews and approves the quality assurance project plan (QAPP), project progress, and deliverables.

TSSWCB – Provides project oversight and funding at the state level.

Mitch Conine, 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 workplan are completed as specified.

Donna Long, TSSWCB QAO
Reviews and approves the QAPP and any amendments or revisions and ensures distribution of approved/revised QAPPs to TSSWCB and USEPA participants. Responsible for verifying that the QAPP is followed by project participants. Determines that the project meets the requirements for planning, quality assurance/quality control (QA/QC), and reporting. Monitors implementation of corrective actions. Coordinates or conducts audits of field and laboratory systems and procedures.

TWRI – Provides the primary point of contact between the TSSWCB and the project contractors. Tracks and reviews deliverables to ensure that tasks in the workplan are completed as specified. Responsible for coordination, review, and delivery of quarterly project reports (QPRs) and the watershed protection plan (WPP). Responsible for maintaining and updating the Buck Creek web site.

B. L. Harris, TWRI Acting Director; Project Coordinator
Responsible for ensuring that tasks and other requirements in the contract are executed on time and as defined by the grant workplan; assessing the quality of work by participants; submitting accurate and timely deliverables and costs to the TSSWCB; and coordinating attendance at conference calls, meetings, and related project activities.

Lucas Gregory, TWRI QAO
Responsible for determining that the QAPP meets the requirements for planning, QA/QC, and reporting activities conducted by TWRI.

AgriLife-El Paso – Provides technical advice to AgriLife Vernon on bacterial analyses and sample collection for bacterial source tracking (BST). Responsible for BST assays, data analysis and interpretation. AgriLife El Paso will contribute to QPRs and the final project report.

Dr. George D. Di Giovanni, Associate Professor, Environmental Microbiology; Lab Manager; Project Co-Leader
Responsible for conducting *Bacteroidales* PCR and *E. coli* ERIC-PCR and RiboPrinting BST analyses to determine the human and animal influence of bacterial loading to the creek and the need to augment the Texas Known Source Library with samples from the Buck Creek watershed. Responsible for technical oversight of activities involved in generating analytical data by the AgriLife Vernon laboratory. Responsible for general facilitation of audits and reporting of corrective actions.

AgriLife Vernon – Responsible for collection of fecal samples, ambient, and storm water samples. Responsible for data analysis. Responsible for the isolation, confirmation, and archival of *E. coli* for BST and pre-processing of water concentrates for the *Bacteroidales* PCR. AgriLife Vernon will contribute to the development of QPRs and the WPP.

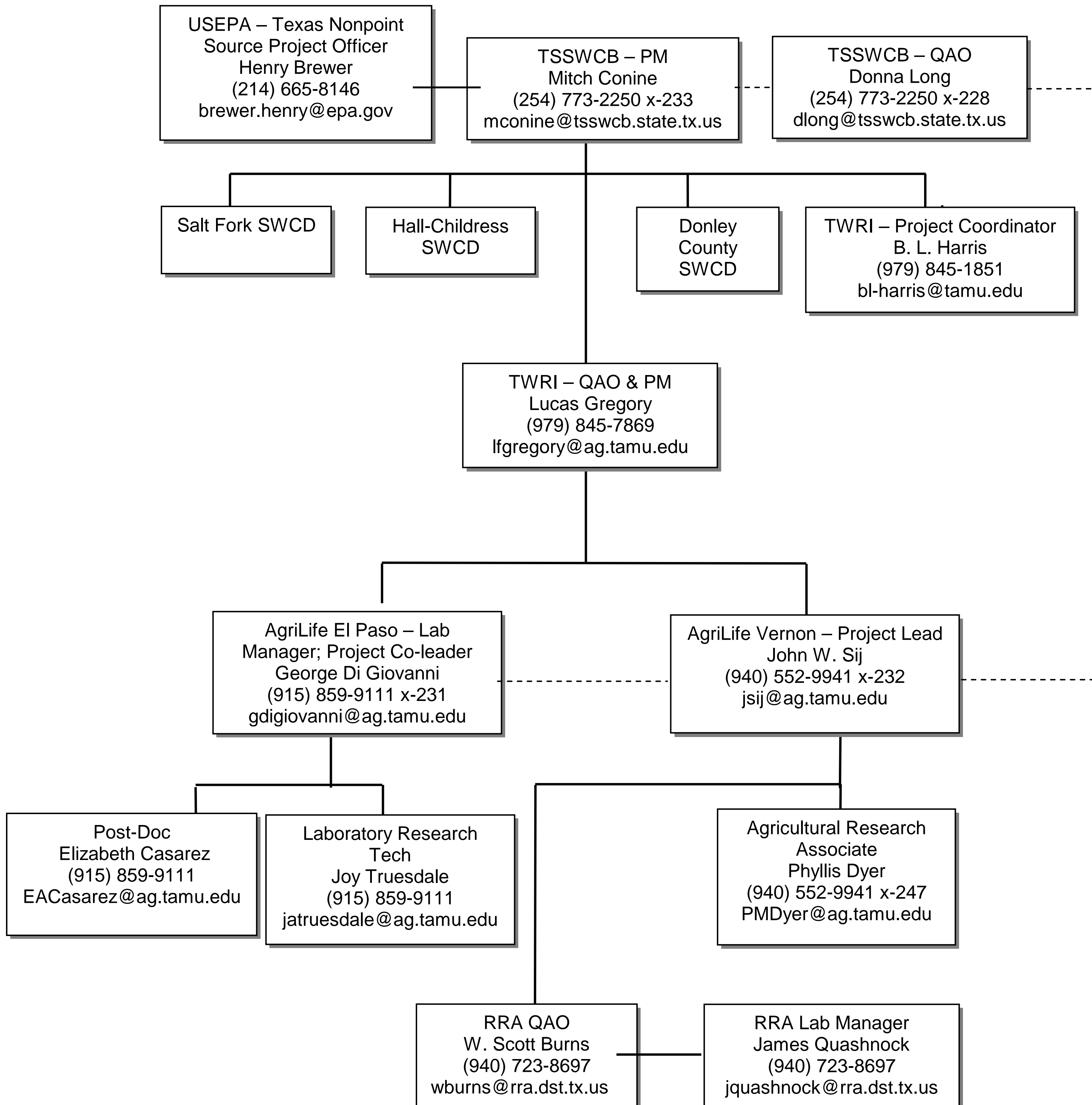
Dr. John W. Sij, Professor; Research Agronomist; Project Leader
Responsible for coordinating and supervising field sampling activities. Responsible for ensuring that field personnel have adequate training, equipment, and a thorough knowledge of standard operation procedures (SOPs) specific to the analysis or task performed and/or supervised. 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 workplan and in the QAPP. Responsible for verifying that the data produced are of known and acceptable quality. Responsible for ensuring adequate training and supervision of all activities involved in generating analytical data for this project. Responsible for news releases, public presentations, and publications including accuracy of data disseminated concerning ongoing activities in the Buck Creek watershed. Responsible for the facilitation of audits and the implementation, documentation, verification, and reporting of corrective actions. Responsible for coordinating and organizing a stakeholder group that serves to steer WPP development, drafting a WPP with direction from stakeholders and submitting it to TSSWCB and USEPA. Responsible for submitting accurate and timely data analyses and other materials for QPRs and final reports to TWRI.

Red River Authority of Texas – Responsible for conducting nitrate analysis of water samples collected by AgriLife Vernon and reporting of those data back to AgriLife Vernon for inclusion in project reports and data sets.

James Quashnock, Laboratory Supervisor
Responsible for ensuring that all samples received in the Environmental Services Division Laboratory are within the allotted time, and that the chain of custody (COC) has been observed. Ensures that the samples are analyzed in accordance with standard accepted methods as described in the SOP manual. The Laboratory Supervisor further ensures that all analysis results are correctly performed and properly recorded on the lab data sheets and in the appropriate analytical log books prior to transmittal to the QAO.

W. Scott Burns, RRA QAO
Responsible for ensuring that the RRA laboratory conforms to approved nitrate analysis methods and ensuring that analysis results are transmitted to AgriLife Vernon in an efficient and timely manner. Responsible for identifying, and maintaining Laboratory QA records. Coordinates and monitors deficiencies, nonconformances, and corrective action for sample analysis conducted at the RRA laboratory. Coordinates and maintains records of data verification and validation at the RRA laboratory. Coordinates the research and review of technical QA material and data related to water quality monitoring system design and analytical techniques utilized by RRA laboratory for this project.

Figure A.4-1. Project Organization Chart



Section A5: Problem Definition/Background

The Red River Basin includes 30 classified segments and 11 major reservoirs that cover 58,773 hectares (145,169 acres). Buck Creek, also known as Spiller Creek, is a small waterbody situated within the Red River Basin. Buck Creek joins the Lower Prairie Dog Town Fork of the Red River (segment 0207) to form the Red River above Pease River (Segment 0206). Buck Creek is located within Ecoregions 26 and 27, Central Great Plains. Small streams within this region are typically characterized by widely varying flows and high levels of dissolved salts, generally originating from saltwater seeps and springs. Buck Creek (segment 0207A) is situated within a predominantly rural and agricultural landscape in the panhandle region of Texas.

Land use in the watershed is predominantly row crops and grasslands (Figure A.5-1). Temperatures in the region range from -4°C to 34°C (25ºF to 93ºF) and rainfall averages approximately 53 centimeters (21 in.) annually. During periods of rainfall, bacteria (*Escherichia coli* specifically) originating from birds, mammals, livestock, inadequately treated sewage, and/or failing septic systems may be washed into streams, potentially impacting recreational use of this waterbody. Bacterial indicators, such as *E. coli,* may remain in the streams at levels exceeding established criteria for recreational use and can be measured well after a rainfall event. These organisms are normally found in feces of warm-blooded animals and are generally not harmful to human health, but may indicate the presence of pathogens that can cause disease.

The State of Texas requires that water quality in Buck Creek be suitable for fishing, swimming, wading, and a healthy aquatic ecosystem. However, data obtained from water quality monitoring indicate that bacterial levels are sometimes elevated in the creek. Although these data points provide an indicator of a potential water quality problem, the data do not provide conclusive evidence of persistent impairment; rather, it suggests a temporal recurring phenomenon. Recent data evaluations conducted by the TCEQ also revealed that, periodically, nitrate levels in the creek exceed the screening level and are, therefore, a concern. This concern justifies the need for more extensive nitrate analysis to better understand the situation and attempt to determine the source of the elevated nutrients.

Like most states, Texas does not directly monitor pathogens because of the difficulty and expense of measuring them. Instead, it tests for the presence of organisms that indicate the likely presence of pathogens. For example, *E. coli* is typically used as a bacterial indicator in the assessment of fresh water quality. These indicators are used to estimate the relative risk of swimming or other recreational activities involving direct contact with the water because the probability of becoming ill is greater when the bacteria counts are elevated.

In August 2001, the TCEQ proposed developing a total maximum daily load (TMDL) for Buck Creek utilizing the data collected through the Texas Clean Rivers Program (CRP). However, because TSSWCB is the lead agency for the State of Texas in abating agricultural nonpoint source (NPS) pollution, the TSSWCB took the lead in Buck Creek. Working closely with the Hall-Childress, Donley County, and Salt Fork Soil and Water Conservation Districts (SWCDs),

RRA, TWRI, Texas AgriLife Research and Extension; TSSWCB's first step was to initiate a CWA §319(h) funded project, *Bacterial Monitoring for the Buck Creek Watershed* (TSSWCB 03-07), to verify the impairment and assess the seasonal levels of *E. coli* throughout the watershed. The existing dataset was very limited, composed of only 14 *E. coli* samples (with 7 excedances of >394 colony forming units (cfus)/100ml) and 20 fecal coliforms (with 12 excedances of >400 cfu/100ml) over the course of 5 years, and represented only one site. From May 2004 to May 2007, *E. coli* levels were monitored at 13 sites throughout the watershed with the addition of two more sites near Site 10 during the last 14 months of study and verified the bacterial impairment in the watershed (Figure A.5-2).

Figure A.5-1. Buck Creek Watershed Land Cover

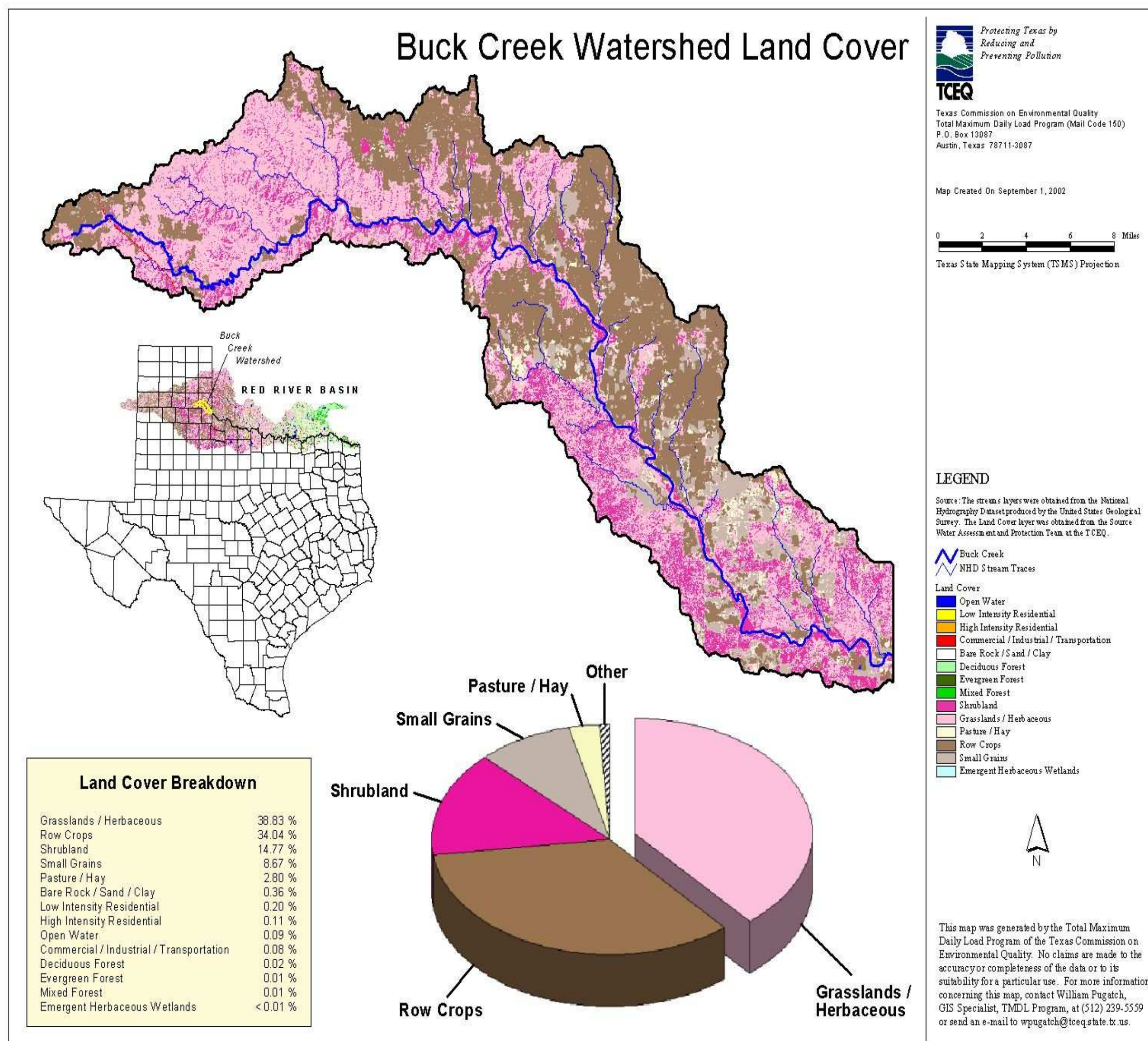
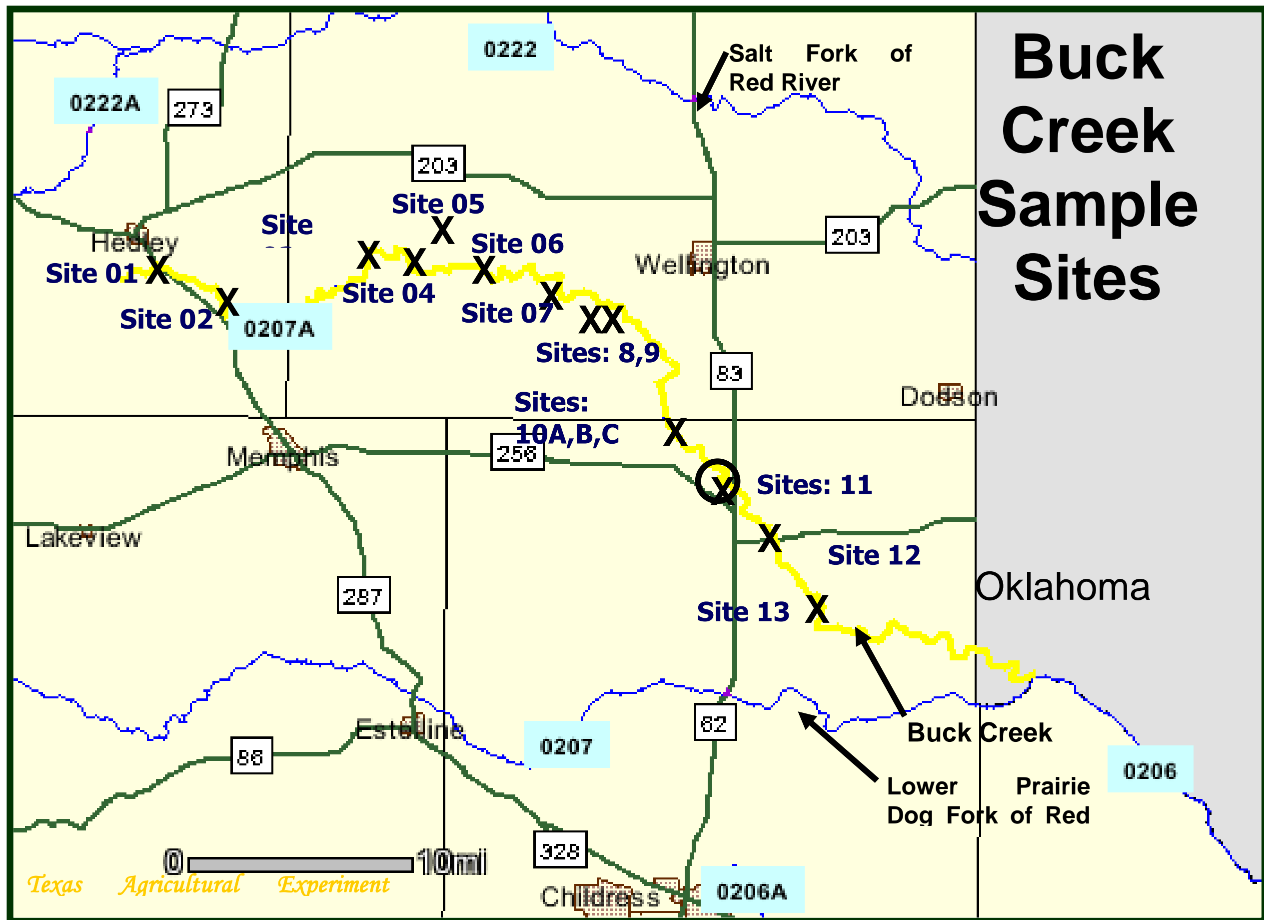


Figure A.5-2. Buck Creek Site Map



The *2008 Texas Water Quality Inventory and 303(d) List* identifies nitrates as a concern for non-attainment for Buck Creek (assessment unit 0207A_01 which is the reach from the Oklahoma state line to House Log Creek). To date, only 11 samples have been collected at the US 83 bridge resulting in insufficient data to adequately assess the geospatial and temporal extent of the elevated nutrients. The current screening level for nitrates in streams is 1.95 mg/L and should not be exceeded more than 25 percent of the time; of the data collected, 10 samples have exceeded the screening level. The approach will be to get a more representative data set for the watershed and to actually determine if nitrates are problematic.

Section A6: Project/Task Description

General Project Description

The seasonal dynamics in fecal bacteria populations in Buck Creek can be the result of a number of landscape utilization processes associated with human habitation, agricultural activities, herbivore and avian guild landscape utilization patterns, and cattle landscape utilization patterns. A proactive WPP for mitigating fecal bacteria impairment will be based on understanding and then altering the timing, frequency, and duration of fecal bacteria loading activities within the riparian zone immediately adjacent to Buck Creek. Currently there is no geospatial inventory of the landscape components in this watershed. Furthermore, there is an information vacuum as to the spatial and temporal distribution of potential sources of fecal bacteria loading. In this project, landscape utilization patterns will be examined to assess their potential role in Buck Creek fecal coliform impairment and evaluate subsequent mitigation strategies.

The *Watershed Protection Plan Development* project (06-11) will basically pick up where the *Bacterial Monitoring for the Buck Creek Watershed* project (TSSWCB Project 03-07) ended. The TSSWCB, TWRI, Texas AgriLife Research and Extension, Hall-Childress, Donley County, and Salt Fork SWCDs, and RRA will work together to (1) identify the specific sources of the bacteria, (2) evaluate alternatives for restoring the waterbody, and (3) develop a WPP to restore the waterbody through a stakeholder driven process.

Identification of Sources

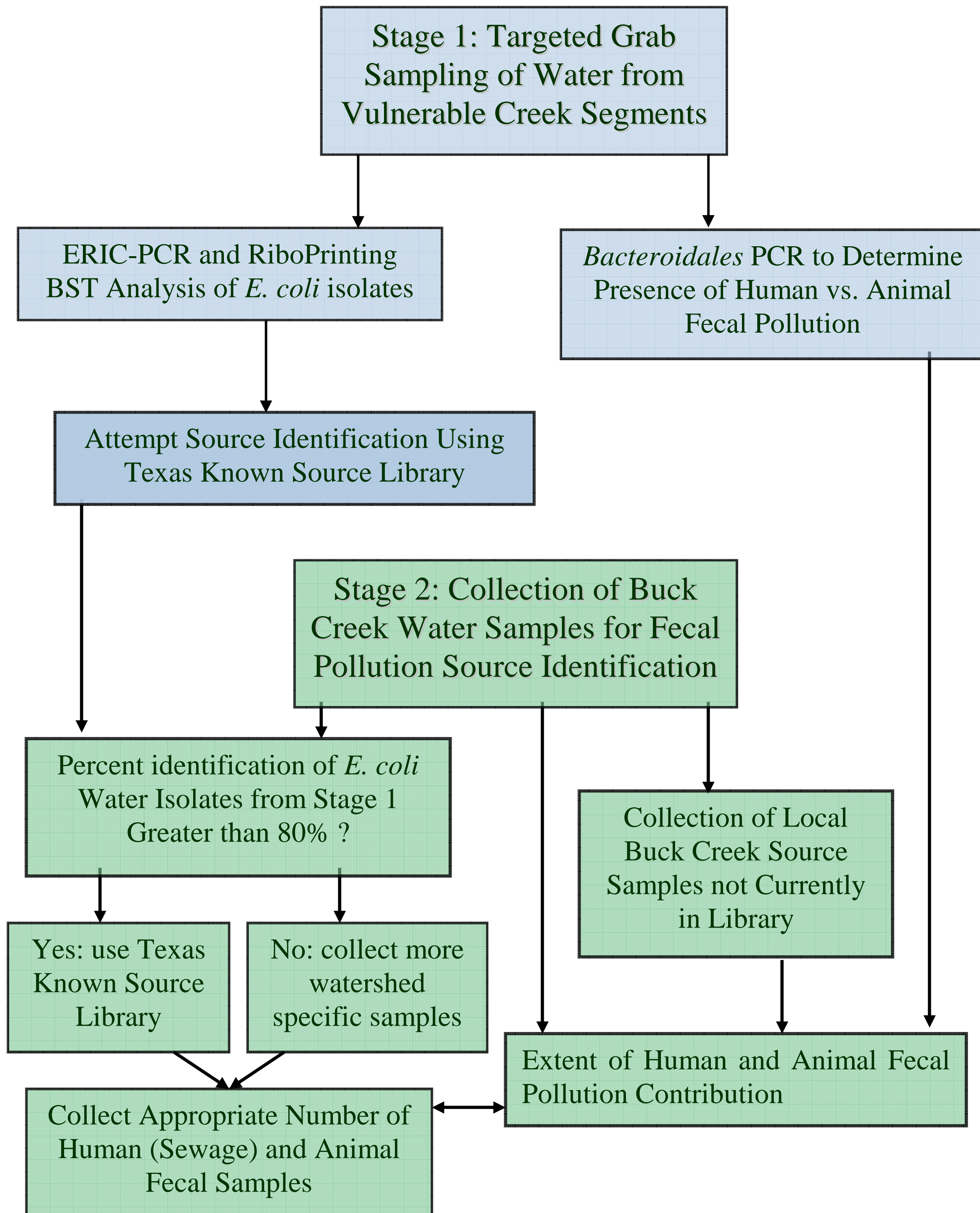
The project will include review and evaluation of existing data and information pertaining to bacterial contributions and sources to Buck Creek. New data, of known and specified quality, will be collected and analyzed to differentiate and quantify the relative contributions of bovine livestock, wildlife, and other human and animal *E. coli* sources. This assessment and differentiation between bacteria sources will utilize, and be coordinated with, the BST Texas Known Source Library maintained by AgriLife El Paso which now contains almost 2,000 *E. coli* isolates from over 1,500 different domestic sewage and animal fecal sources. The library contains diverse *E. coli* isolates that were selected after screening over 4,400 isolates by genetic fingerprinting to exclude identical isolates from the same sample and include isolates with unique genetic fingerprints. This project will provide sufficient documentation of the data and technical analyses conducted that will aid the project staff in communicating the assessment results to watershed stakeholders, TSSWCB, TCEQ, and USEPA, as necessary.

Livestock and wildlife fecal samples, along with septic system samples will be obtained from a variety of sources in the watershed. Sources will be identified through a sanitary survey to be conducted by AgriLife Vernon. An inventory of existing land use patterns in the Buck Creek watershed will be conducted utilizing available imagery and aerial observation. The locations of bridges, springs, tributaries, and human habitation will be ground-truthed to ensure high quality data.

BST for this project will be conducted in two stages. Stage 1 involves assessment and monthly targeted grab sampling of creek segments at greatest risk for fecal pollution loading for a period of six months. Stage 1 will include analysis of water samples for *E. coli* as previously performed by AgriLife Vernon using USEPA Method 1603 [*USEPA/821/R-02/023. September 2002. Escherichia coli in Water by Membrane Filtration Using Modified Membrane-Thermotolerant Escherichia coli (modified m-TEC) Agar*]. In addition, a PCR genetic test for the *Bacteroidales* fecal bacteria will be performed by AgriLife El Paso to determine if creek segments are being impacted by human or animal fecal pollution.

Approximately 50 *E. coli* isolates from 50 different water samples will be analyzed using the Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) and RiboPrinting BST methods described below and compared with isolates from the previously developed Texas Known Source Library to determine the need to augment the Library with samples from the Buck Creek watershed. Similarly, water samples (50) collected at the same time as the water samples for *E. coli* will be analyzed for *Bacteroidales* PCR markers (general, human, ruminant, swine, and equine). Depending on the results of the Stage 1 BST work, the number of water and source isolates and the types (sewage or animal) of source samples listed below for Stage 2 may change. That is, if it is determined that local augmentation of the Library is needed, more source samples and fewer water samples will be analyzed. Conversely, if it appears that the existing Texas Known Source Library is suitable for identification of water isolates, more water samples and fewer Buck Creek source samples may be analyzed. This will allow for the most useful BST results to be provided within funding limitations. Stage 2 of the BST work will involve augmentation of the Library with local Buck Creek *E. coli* from known sewage and animal sources and BST analysis of *E. coli* isolated from water samples, with the sampling design dependent on the results of the Stage 1 BST assessment. An experimental approach flow diagram is presented in Figure A6-1.

Figure A.6-1. Flow Diagram of Experimental Approach for BST



AgriLife Vernon will be responsible for collecting, processing, and isolating *E. coli* from water and fecal samples and for pre-processing of water samples for *Bacteroidales* PCR. *E. coli* will be isolated from the samples using standard microbiological methods as previously used in TSSWCB and TCEQ BST projects. *E. coli* will be isolated from water samples using USEPA Method 1603 and modified membrane Thermotolerant *E. coli* (mTEC) medium. Counts of E. coli obtained using this method can be used for regulatory purposes as well. Fecal specimens or domestic sewage samples will also be streaked (resuspended in buffer if necessary) onto modified mTEC medium. The use of modified mTEC medium for isolation of *E. coli* from both water and source samples will help avoid selection of different types of E. coli due to different media. Inoculated plates will be incubated at 35±0.5°C for 2 hours to resuscitate stressed bacteria, then incubated at 44.5±0.2°C for approximately 20 to 24 hours. The modified mTEC method is a single-step method that uses one medium and does not require testing using any other substrate. The modified medium contains the chromogen 5-bromo-6-chloro-3-indolyl-β-D-glucuronide (Magenta Gluc), which is catabolized to glucuronic acid (a red/magenta-colored compound) by *E. coli* that produces the enzyme β-D-glucuronidase. This enzyme is the same enzyme tested for using other substrates such as the fluorogenic reaction with 4-methylumbelliferyl-β-D-glucuronide (MUG) observed by ultraviolet light (UV) fluorescence. *E. coli* colonies from the modified mTEC medium will be picked and streaked for purity on nutrient agar with MUG (NA-MUG) to confirm glucuronidase activity and culture purity. Cultures of selected isolates will be archived using glycerol freezing medium (-80°C). AgriLife Vernon will ship bacterial cultures to AgriLife El Paso for BST analyses. In addition, AgriLife Vernon will be responsible for collecting water samples from selected sampling sites and nearby water wells that will be analyzed by the RRA for nitrates. Specific sites/water wells to be monitored will be based on 1) landowner cooperation, 2) proximity to the creek and 3) depth to water. These sites will be identified specifically through QAPP amendments as described in section A9.

E. coli source isolates will be selected using an approach to maximize the diversity of strains represented in the library. AgriLife El Paso will screen confirmed *E. coli* bacterial colonies using ERIC-PCR. ERIC-PCR will be used to identify unique *E. coli* isolates from each sample and eliminate further analysis of identical isolates (clones). At least one *E. coli* isolate from each fecal or wastewater sample will be included in the library, even if it is identical to a previously isolated *E. coli*. Therefore, abundant/common strains will be sufficiently represented in the libraries. It is anticipated that over 300 *E. coli* colonies from approximately 100 source samples will be screened by ERIC-PCR. Approximately 100 of those isolates will be selected for automated ribotyping (RiboPrinting) BST analysis and inclusion in the source identification library. AgriLife El Paso will simultaneously analyze *Bacteroidales* fecal bacteria to determine the impacts of human vs. animal fecal contamination into the creek.

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 barcode patterns. 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') and has a throughput of 32 isolates per day.

The *Bacteroidales* PCR method is a culture-independent molecular method which targets genetic markers of *Bacteroidales* and *Prevotella* spp. fecal bacteria that are specific to humans, ruminants (including cattle and deer), pigs, and horses [Bernhard, A. E. and K. G. Field (2000). "A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA." Appl Environ Microbiol 66(10): 4571-4574**;** Dick, L. K., A. E. Bernhard, et al. (2005). "Host distributions of uncultivated fecal Bacteroidales bacteria reveal genetic markers for fecal source identification." Appl Environ Microbiol 71(6): 3184-3191]**.** The method has high specificity and moderate sensitivity [Field, K. G., E. C. Chern, et al. (2003). "A comparative study of culture-independent, library-independent genotypic methods of fecal source tracking." J Water Health 1(4): 181-94]. For this method, 100 ml water samples are concentrated by filtration, DNA extracted from the concentrate and purified, and aliquots of the purified DNA analyzed by PCR. For pre-processing of water samples for *Bacteroidales* PCR, AgriLife Vernon will filter the water samples, place the filters in DNA lysis buffer and freeze at -80° C until shipped to AgriLife El Paso for analysis. AgriLife El Paso will continue with DNA extraction and purification. DNA extracted from the water samples will be tested for the general, human, ruminant (including cattle and deer), pig (including feral hogs), and horse fecal markers. Results are typically expressed as presence/absence of the host-specific genetic markers; therefore, this method is not quantitative. However, quantitative PCR (qPCR) detection of the markers will also be performed by AgriLife El Paso. It must be emphasized that the *Bacteroidales* qPCR analysis will provide only *semi-quantitative* data for the markers. These semi-quantitative data will be used for a trend analysis of *individual* marker abundance (low, medium, and high) over time at sampling sites. Presently, these semi-quantitative data cannot be used to calculate fecal loadings for different sources due to uncertainties that need to be addressed by the BST scientific community including differences in marker concentration in feces from different animal sources, disparities in detection sensitivity for the markers, and differential persistence of the markers in the environment. *Bacteroidales* qPCR reaction mix will contain the dye SYBR Green I which fluoresces when bound to double stranded DNA (dsDNA). During each cycle of the PCR, SYBR Green binds to dsDNA PCR product (amplicons), resulting in an increase in reporter fluorescence detected by the instrument. The threshold cycle (C_T) is the fractional PCR cycle number at which a significant increase in signal fluorescence above baseline is first detected for a sample by the instrument software. The SYBR Green fluorescence signal increases in direct proportion to the concentration of the PCR product being formed, with lower C_T values indicating earlier amplification and higher starting amounts of target in the samples.

A total of 500 *E. coli* isolates obtained from ambient water samples will be characterized using ERIC-PCR and RiboPrinting. DNA patterns of those isolates will be compared to the Buck Creek augmented Texas Known Source Library of over 1,900 *E. coli* isolates from known animal and human sources collected throughout Texas. Water isolates will be identified to cattle, other

livestock, avian and non-avian wildlife, domestic sewage, and pet sources (six-way split), as well as a broader three-way split of livestock, domestic sewage and wildlife.

Nitrate analysis will also be conducted. Nitrates have been identified as a concern for non-attainment. Initially, nitrate samples will be collected at all sampling sites where routine water quality samples will be collected. These samples will be collected for 12 months to determine if there are seasonal and temporal variations in nitrate levels. If it is determined that nitrates are not a concern, sampling will cease. However, if it is determined that nitrates are problematic in the watershed, sampling will continue in an effort to identify the source of the nitrates. Samples will be collected by AgriLife Vernon and analyzed by RRA.

The project plan milestones for the project/task description of this section of the study are summarized in Table A.6-2. The expectation for completing the associated tasks described in Section A6 is September 2010.

Table A.6-2. Project Plan Milestones

TASK	PROJECT MILESTONES	AGENCY	START	END
1.1	Quarterly meetings (when necessary) including project participants, landowners and other interested parties.	AgriLife Vernon, TWRI	Oct 06	Sept 10
1.2	Prepare QPRs submitted electronically to TSSWCB and distributed to project cooperators and participants.	AgriLife Vernon, TWRI	Oct 06	Sept 10
2.1a	Develop DQOs and QAPP for review by USEPA.	AgriLife Vernon, TWRI	Oct 06	Mar 07
2.1b	Approve QAPP.	TSSWCB & USEPA	Mar 07	May 07
4.1	AgriLife Vernon will perform routine sampling (grab sampling) at six locations (Sites 3, 5, 6, 10a, 10c, 11) on Buck Creek	AgriLife Vernon	Mar 07	Sept 10
4.2	Collect a minimum of 8 rain event grab samples (or, if less than 8, the maximum number of events for project duration)	AgriLife Vernon	Mar 07	Sept 10
4.3	Perform quarterly sampling (grab sampling) at 2 flowing tributary sites within the Buck Creek watershed when flow is present. Tributary 1 and Site 5	AgriLife Vernon	Mar 07	Sept 10
4.4	Compile and analyze the sampling data.	AgriLife Vernon	Mar 07	Sept 10
5.1	BST will be used to assess water samples, including rainfall event samples, from Buck Creek to determine the need to augment the Library with samples specific to the watershed and determine the presence of human and animal fecal pollution. Approximately 50 *E. coli* isolates from water samples will be analyzed using ERIC-PCR and RiboPrinting. *Bacteroidales* PCR will be used to analyze 50 water samples for human and animal fecal pollution markers.	AgriLife Vernon, AgriLife El Paso	Mar 07	Sept 07
5.2	Known source samples (approx. 100) will be collected. *E. coli* will be cultured, isolated, and analyzed using ERIC-PCR to select isolates for RiboPrinting and addition to the source identification library	AgriLife Vernon, AgriLife El Paso	Sept 07	Sept 10
5.3	Ambient water sampling, analysis and isolation of *E. coli* will be conducted by AgriLife Vernon. Samples will be enumerated and *E. coli* will be isolated, the cultures will be archived, and isolates will be sent to AgriLife El Paso for ERIC-PCR and RiboPrinting BST analysis and source identification.	AgriLife Vernon, AgriLife El Paso	Sept 07	Sept 10
5.4	ERIC-PCR and RiboPrinting fingerprints of approximately 500 *E. coli* isolates from ambient water samples will be analyzed and compared to source library isolates using Applied Maths BioNumerics software.	AgriLife El Paso	Sept 07	Sept 10
5.5	AgriLife El Paso will compile and analyze the BST data. Water isolates will be sourced to cattle, other livestock, avian and non-avian wildlife, domestic sewage, and pet sources. Results will help identify the sources needed to be addressed by the WPP.	AgriLife El Paso	Mar 07	Sept 10

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. One objective is to identify specific sources of bacteria and nitrates entering Buck Creek. A second objective is to monitor micro-watersheds through data collection and analysis, and provide data to inform SWCD's, stakeholder committee, and landowners of any potential or existing water quality issues and/or problems. Achievement of these objectives will support decisions for implementation of appropriate best management practices (BMPs) in order to reduce fecal bacteria levels in the Buck Creek watershed to comply with existing water quality standards.

Following are actions that will be undertaken by this project to assess bacterial pollution within Buck Creek Watershed:

- Monitor water quality as related to bacterial pollution in Buck Creek by in-stream water sampling
- Determine the source of the bacterial impairment using BST

The measurement performance criteria to support the project objective are specified in Table A.7-1.

When sufficient flow (above 7Q2 or 0.1 cfs) is present, routine grab samples will be collected on a monthly basis. During routine sampling measurements of dissolved oxygen (DO), conductivity, pH, salinity, stream flow, and water temperature will be obtained *in situ*. Water samples will be analyzed for *E. coli.*

Water samples will also be collected and transported to the RRA for nitrate analysis. AgriLife Vernon delivers water samples to RRA for nitrate analysis using USEPA method 300.0. Appropriate DQOs and QA/QC requirements for this analysis are reported in Tables A.7-1 and B.2-1. The nitrate samples will be picked up along with these samples and will be delivered to the RRA lab within 48 hours of sample collection. Results will then be reported by RRA back to AgriLife Vernon for inclusion in project data sets.

Table A.7-1. Data Quality Objectives for Measurement Data

NA = Not applicable; mg/L = milligrams per liter; col = colonies; mL = milliliters; m/s = meters per second; µS/cm = microsiemens per centimeter; ft = feet; m = meters; °C = degrees Celsius

Parameter	Units	Method Type	Method	Method Description	Parameter Code	AWRL[1]	Precision of Laboratory Duplicates	Accuracy[2]	Precision (RPD of LCS/ LCSD)	Percent Complete[3]
Field Parameters:										
Days Since Last Significant Precipitation	Days	Observation	TCEQ SOP V-1	Field observation	72053	NA	NA	NA	NA	90
Flow Severity	1-no flow 2-low 3-normal 4-flood 5-high 6-dry	Visual Observation	TCEQ SOP V-1	Field observation	01351	NA	NA	NA	NA	90
Flow	cfs	Handheld meter	TCEQ SOP V-1	Automated Instrument	00061	NA	NA	NA	NA	90
Flow Method	1-gage 2-electric 3-mechanical 4-weir/flume 5-Doppler	Handheld meter	TCEQ SOP V-1	Automated Instrument and Calculation	89835	NA	NA	NA	NA	90
Water Level	m	Manual measurement	USGS	Meter stick	NA	NA	NA	NA	NA	90
Water Temperature	˚C	Handheld meter	USEPA 170.2	Automated Instrument	00020	0.2	NA	±0.25	NA	90
Specific Conductance	µS/cm	Handheld meter	SM 2510-B	Automated Instrument	00094	20 µS/cm	NA	±2% of range	NA	90
Dissolved Oxygen	mg/L (ppm)	Handheld meter	USEPA 360.1	Automated Instrument	00300	2.0	NA	±0.2	NA	90
pH	pH units	Handheld meter	USEPA 150.1	Automated Instrument	00400	0.2	NA	±0.2	NA	90

Table A.7-1. continued

Parameter	Units	Method Type	Method	Method Description	Parameter Code	AWRL [1]	Precision of Laboratory Duplicates	Accuracy [2]	Precision (RPD of LCS/ LCSD)	Percent Complete [3]
Lab Parameters:										
Salinity	ppt	Handheld meter	SM 2520-B	Automated Instrument	00480	0.01 ppt	NA	±0.01	NA	90
Nitrate/nitrite – N, total	mg/L	Ion Chromatograph	USEPA 300.0	Automated Instrument	00630	.05	NA	NA	20	90
E. coli in water	CFU/ 100 mL	Membrane filter culture on modified mTEC agar	USEPA 1603	Membrane Filter	31648	1	3.27* ΣRlog/n	NA	NA	90
E. coli RiboPrint fingerprint	NA	DNA/ image matching	AgriLife El Paso SOP	RiboPrinting	NA	NA	90% identical [4]	90% correct [4]	NA	90
E. coli ERIC-PCR fingerprint	NA	DNA/ image matching	AgriLife El Paso SOP	ERIC-PCR	NA	NA	90% identical [4]	90% correct [4]	NA	90
Bacteroidales PCR and qPCR	Qualitative or semi-quantitative for marker abundance	PCR presence or absence; qPCR (semi-quantitative)	AgriLife El Paso SOP	*Bacteroidales* PCR and qPCR	NA	NA	100% agreement for presence/ absence detection of markers; less than 10% relative standard deviation for qPCR [4,5]	90% correct (presence/absence and low, medium, high marker abundance for qPCR) [4]	NA	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
[5] For *Bacteroidales* qPCR, relative standard deviation of cycle threshold (C_T) values will be used to assess precision

Ambient Water Reporting Limits (AWRLs)
The AWRL establishes the reporting specification at or below which data for a parameter must be reported based on given freshwater screening criteria. The AWRLs specified in Table A7.1 are the program-defined reporting specifications for each analyte and yield data of acceptable quality for assessment.

Precision
The precision of laboratory data is a measure of the reproducibility of a result from repeated analyses. It is strictly defined as a measure of the closeness with which multiple analyses of a given sample agree with each other. Precision is assessed by repeated analyses of a sample. 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.

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

The 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.

Field splits are used to assess the variability of sample handling, preservation, and storage, as well as the analytical process, and are prepared by splitting samples in the field. Control limits for field splits are defined in Section B5.

The ERIC-PCR and RiboPrinting BST techniques are qualitative assays, generating two different types of DNA fingerprints. For *Bacteroidales* there is a presence absence qualitative PCR assay and a semi quantitative qPCR assay. Precision for ERIC-PCR and RiboPrinitng will be determined using a control strain of *E. coli* (QC101), while fecal DNA from known-source samples will be used for *Bacteroidales* PCR. For ERIC-PCR and RiboPrinitng, the data quality objective is 90% precision. For *Bacteroidales* PCR the data quality objective is 100% agreement in marker detection among replicates. For *Bacteroidales* qPCR the data quality objective is less than 10% relative standard deviation (RSD) for threshold cycle (C_T) values.

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 A.7-1.

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

Accuracy for BST methods will be assessed using *E. coli* (QC101) for ERIC-RP and fecal DNA from known-source samples for *Bacteroidales* PCR. For the *E. coli* methods the data quality objective is 90% accuracy for correct identification to library strain or source. For *Bacteroidales* PCR, the data quality objective is 90% accuracy for the presence/absence of the appropriate

markers in known source fecal DNA samples. For *Bacteroidales* qPCR, dilutions of known source fecal DNA samples will be used and the data quality objective is for 90% accuracy of classification into low, medium, and high marker abundance groups for replicates.

Bias

Bias is a statistical measurement of correctness and includes multiple components of systematic error. A measurement is considered unbiased when the value reported does not differ from the true value. Bias is determined through the analysis of laboratory control samples prepared with verified and known amounts of all target analytes in the sample matrix and by calculating percent recover. Results are compared against measurement performance specifications and used during evaluation of analytical performance.

Sensitivity

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

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

For analyzing environmental samples using PCR, the detection limits can be for the limit of the entire method or the limit of the PCR procedure. For bacteria, protozoa, and fungi these limits are often measured in terms of the minimum detectable counts or CFUs.

Representativeness

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

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

Completeness
The completeness of the data is a measure of how much of the data is available for use compared with the total potential data. Ideally, 100% of the data would be available. However, the possibility of unavailable data due to accidents, weather, insufficient sample volume, broken or lost samples, etc. is to be expected. Therefore, it will be a general goal of the project(s) that 90% data completion is achieved. Should less than 90% data completeness occur, the TWRI PM will initiate corrective action. Data completeness will be calculated as a percent value and evaluated with the following formula:

% completeness = (SV X 100) / ST

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

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. AgriLife Vernon 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.

The AgriLife Vernon laboratory is also in the process of becoming National Environmental Laboratory Accreditation Conference (NELAC) certified for USEPA method 1603. This method is a quantitative method used to analyze *E. coli* and yields a direct count of bacteria in water based on the development of bacteria colonies that grow on the surface of the membrane filter. This method consists of filtering a water sample thru a membrane that retains the bacteria, placing the membrane on a modified mTEC agar, incubated at 35±0.5°C for 2 hours to resuscitate the injured or stressed bacteria, and then incubated at 44.5±0.2°C for 22 hours. This modified method eliminates the transfer of the membrane filter to another substrate. The target colonies on modified mTEC agar are red or magenta in color after the incubation period.

As a part of the NELAC approval process, the AgriLife Vernon Lab had to select a stand-alone name for their facility. The name selected is the “Texas AgriLife Research Water Quality Laboratory.” At this time, the lab is only seeking NELAC accreditation for USEPA method 1603.

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 for at least five years. In addition, AgriLife Vernon 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, a blank COC form is presented in Appendix B, and blank field data reporting forms are presented in Appendix C.

QPRs will note activities conducted in connection with the water quality monitoring program, 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.

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 nonconformances; 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)

One main goal of this project is to organize a diverse stakeholder group that participates in the development of a WPP for the Buck Creek watershed. The other primary goal of this project is to continue monitoring subwatersheds through data collection and analysis, and provide data to inform SWCDs and landowners of any potential or existing water quality issues and/or problems. In addition, water samples will be analyzed to determine the source of bacteria entering the stream. This information will be instrumental in evaluating potential BMPs to implement in the watershed as well as aid in WPP development. Achievement of these objectives will support decisions on how to best target management measures to reduce fecal bacteria levels in the Buck Creek watershed. The waterborne constituents that will be measured are shown in Table B1-1.

Table B1-1. Waterborne Constituents

Parameter	Status	Reporting Units
Laboratory Parameters		
Nitrates	Critical	milligrams per liter (mg/L)
Escherichia coli	Critical	cfu/100ml
Field Parameters		
Dissolved Oxygen	Non-critical	milligrams per liter (mg/L)
Potential Hydrogen (pH)	Non-critical	pH standard units
Specific Conductance	Non-critical	microsiemens per centimeter (µS/cm)
Water Temperature	Non-critical	degrees Celsius (°C)
Salinity	Non-critical	parts per thousand (ppt)
Water Level	Non-critical	Meters (m)
Flow	Critical	cubic feet per second (cfs)
Flow Severity	Critical	1-no flow, 2-low, 3-normal, 4-flood, 5-high, 6-dry

The sampling program is designed to characterize water quality of both base and high flow conditions in Buck Creek and its tributaries. Water quality grab samples will be collected at monthly intervals for all constituents. Routine grab samples will be scheduled for collection on monthly basis but will only be taken if water is flowing at sampling sites. Sampling locations are described in Table B.1-2. Physical parameters that will be measured *in situ* during routine sampling and include flow rate, specific conductance, DO, pH, salinity, and water temperature. Sites that are dry or with pooled water will be noted on the field data sheet and not sampled. Water quality samples collected as part of the routine sampling schedule will be analyzed for bacteria. Additional water samples and field blanks will be collected and sent to RRA for nitrate analysis at these same locations.

In order to obtain representative results, ambient water sampling will occur on a routine schedule over the course of 24 months, capturing dry and runoff-influenced events at their natural frequency. There will be no prejudice against rainfall or high flow events, except that the safety of the sampling crew will not be compromised in case of lightning or flooding; this is left up to the discretion of the sampling crew.

Storm water sampling will occur at the sampling sites listed in Table B.1-2, if accessible, during or after 8 separate rainfall events if they occur during that course of the project. Safety will be the primary concern when collecting these samples. If the research technician feels that their safety is in jeopardy, they will not collect samples.

In the instance that a sampling (Table B.1-2.) site is inaccessible, no sample will be taken and will be documented in the field notebook. If, near the end of the study, the TSSWCB PM/QAO agrees that the sampling has not achieved good representativeness of typical conditions, the final sampling event(s) may be restricted to target a particular environmental condition (e.g., rainfall).

Table B.1-2. Buck Creek Sampling Site Locations

CR= County Road; FM= Farm to Market Road; SH= State Highway; Cnty= County; Lat=Latitude; Long= Longitude

Site	TCEQ Station ID	Subwatershed & General Location	° Lat.	North	° Long.	West
BC-03	20365	CR 40; Collingsworth Cnty	34	51' 25.47"	100	28' 00.93"
BC-05	20367	FM 1056; Collingsworth Cnty	34	51' 50.00"	100	22' 48.10"
BC-06	20368	CR 110; Collingsworth Cnty	34	50' 33.04"	100	20' 46.70"
BC-10A	20371	SH 256; Scrivner Ranch; Childress Cnty	34	43' 46.40"	100	13' 41.00"
BC-10C	20373	SH 256; Scrivner Ranch; Childress Cnty	34	43' 07.80"	100	12' 27.20"
BC-11	15811	US 83; Childress Cnty	34	42' 08.60"	100	11' 19.50"
BC Trib 1	NA	1 mile ESE of the intersection of CR 40 and CR U, Collingsworth Cnty	34	52' 14.60"	100	27' 13.79"

Lat. and Long. are reported in minutes (') and seconds(")

Collection of fecal material samples from known sources will also be done and will be used to validate the BST methodologies. Approximately one hundred known source samples 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. Human sources are from specific areas, but will be selected based on cooperation of the individuals. Therefore; specific global positioning system (GPS) coordinates cannot be listed for sample collections of this nature.

Section B2: Sampling Method Requirements

Water Samples

Typically, water samples will be collected directly from the stream (midway in the stream channel) into sterile wide-mouthed polypropylene bottles or bags. All sample containers will be labeled with the following information:

- collection date
- collection time
- sample location
- and sampler's initials

Care will be exercised to avoid the surface microlayer of water, which may be enriched with bacteria and not representative of the water column. In cases where, for safety reasons, it is inadvisable to enter the stream bed, and boat access is not practical, staff will use a clean bucket and rope from a bridge to collect the samples from the stream. If a bucket is used, care will be taken to avoid contaminating the sample. Specifically, technicians must exert care to ensure that the bucket and rope do not come into contact with the bridge. The bucket must be thoroughly rinsed between stations. Buckets are also to be sanitized between sampling stations with a bleach- or isopropyl alcohol-soaked wipe. The first bucketful of water collected from a bridge is used to rinse the bucket. Rinse water is not returned to the stream, but is instead disposed of away from the sampling site to ensure that the collected sample will not be affected by the bleach or alcohol residual. Samples are collected from subsequent buckets of water. This type of sampling will be noted in the field records.

Water temperature, stream flow, pH, specific conductivity, salinity, and DO will be measured and recorded *in situ* with a multiprobe whenever samples are collected. All field measurements will be conducted in accordance with the methods listed in Table B.4-1. Measurements will only be taken if water is flowing. If a site is not flowing but pooled or dry, that will be noted on the field data sheet. All samples will be transported in an iced container to the laboratory for analysis.

Table B.2-1. Container Types, Preservation Requirements, Temperature, Sample Size, and Holding Time Requirements.

Parameter	Matrix	Container	Preservation	Temperature	Sample Size	Holding Time
nitrates	water	sterile plastic container	none	4°C	125 ml	48 hours
E. coli	water	sterile plastic bag	none	4°C	125 ml	6 hours[1]
E. coli water and fecal isolates	NA-MUG agar	Petri dish 100mm x 15mm	20% glycerol; 80% tryptic soy broth	44.5°C	5 colony streaks	20 – 24 hrs, then frozen
Fecal specimen	feces	sterile plastic bottle placed in biohazard bag	none	4°C	30 g	3 days for *E. coli*, 7 days for *Bacteroidales*
Bacteroidales	Supor filters	15 ml centrifuge tube	GITC buffer	4°C	100 ml	6 hours[1]

[1] 6 hours to deliver to laboratory. In the case that this 6-hour holding time is not met, the *E. coli* quantitative count will be flagged and not reported, though the *Bacteroidales* PCR will still be valid.

Documentation of Field Sampling Activities

Field sampling activities are documented on field data reporting forms as presented in Appendix C. Field observations (flow severity and days since last significant precipitation) are based on SOPs in the *TCEQ Surface Water Quality Monitoring Procedures, Volume 1: Physical and Chemical Monitoring Methods for Water, Sediment, and Tissue* (December 2003). All sample information will be logged into a field log. The following will be recorded for all water sampling:

- station ID
- location
- sampling time
- date
- water depth
- flow rate
- sample collector's name/signature

Detailed observational data are recorded including water appearance, weather, biological activity, stream uses, unusual odors, specific sample information, days since last significant rainfall, estimated hours since rainfall began (if applicable), and flow severity.

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.

Failures in Sampling Methods Requirements and/or Deviations from Sample Design and Corrective Action

Examples of failures in sampling methods and/or deviations from sample design requirements include but are not limited to such things as sample container problems, sample site considerations, etc. Failures or deviations from the QAPP are documented on the field data reporting form and reported to the AgriLife Vernon Project Leader. The AgriLife Vernon Project Leader will determine if the deviation from the QAPP compromises the validity of the resulting data. The AgriLife Vernon Project Leader, in consultation with the TSSWCB QAO will decide to accept or reject data associated with the sampling event, based on best professional judgment. The resolution of the situation will be reported to the TSSWCB in the QPR.

Fecal Sampling Method Requirements

Fecal samples will be obtained one of three ways: 1) using approved Texas Parks and Wildlife methods of capture and release of animals in cages with two exit doors. [This will give technicians the opportunity to see the animal and know that the feces are absolutely from that species. Photos of the animal in the cage and location will be noted on field sheets. The animal will be released safely and once clear of the area, technicians will collect approximately 30 grams of feces (Table B.2-1)]; 2) collecting fecal samples from areas where animals were visually observed defecating by technician; i.e. deer or feral hogs at feeders; and 3) gut samples collected from animals recently killed by cars (within 24 hours) or legally harvested by hunters who have agreed to work with the technician. Gut samples will be collected by using sterile loops inserted anally or by cutting into the intestine using a sterile scalpel.

AgriLife Vernon has gained permission from the Texas Parks and Wildlife Department and Texas A&M University for trapping and has copies of approved permits on file at the AgriLife Vernon lab. The AgriLife Vernon Research Technician also alerts local game wardens of intended trapping activities. At no time will animals be harvested for the sole purpose of this study. If injury occurs to an animal during trapping procedures proper authorities will be contacted and the decision to free the animal or euthanize it to prevent suffering will be solely up to the attending animal officer, game warden, or TPWD personnel.

Documentation of Field Sampling Activities

All samples will be collected in approved specimen containers with spoon attached to the inside of the screw on lid. Specimen container will be labeled with:

- Date
- Time
- Location
- Species
- Samplers initials

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 to AgriLife Vernon.

After releasing animals from the trap and collecting a fecal sample, the cage will be cleaned and moved to prevent possible cross contamination of subsequent fecal samples. Traps will be tripped on the last work day of the week to prevent animals from being confined in cages more than 10 hours (overnight). Traps will be reset the first day of the week samples are to be collected and checked daily or more often during periods of high animal activity. Traps will be set in shaded areas near the creek to reduce heat stress on the animals and for their safety. During periods of high temperature, trapping may be rescheduled.

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 water, nitrate and 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. The resolution of the situation will be reported to the TSSWCB in the QPR. The CARs will be maintained by the TSSWCB PM.

Section B4: Analytical Methods Requirements

E. coli in water samples will be isolated and enumerated by laboratory personnel using modified mTEC agar, USEPA Method 1603 [*USEPA/821/R-02/023. September 2002. Escherichia coli in Water by Membrane Filtration Using Modified Membrane-Thermotolerant Escherichia coli (modified m-TEC) Agar*]. The modified mTEC method is a single-step method that uses one medium and does not require testing using any other substrate. The modified medium contains a chromogen, 5-bromo-6-chloro-3-indolyl-ß-D-glucuronide, which is catabolized to glucuronic acid and a red- or magenta-colored compound by *E. coli* that produce the enzyme ß-D-glucuronidase. This enzyme is the same enzyme tested for using the MUG substrate and UV fluorescence in other *E. coli* assays. A complete listing of methodology used to analyze water and fecal samples for bacteria is given in Appendix D.

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.

Table B.4-1. Laboratory Analytical Methods

Parameter	Method	Equipment Used
Laboratory Parameters		
nitrates	USEPA 300.0	Ion Chromatograph
Escherichia coli	USEPA 1603	Filtration apparatus, incubator
E. coli RiboPrint fingerprint	AgriLife EL Paso SOP	RiboPrinter
E. coli ERIC-PCR fingerprint	AgriLife EL Paso SOP	PCR thermal cycler, gel electrophoresis apparatus
Bacteroidales PCR	AgriLife EL Paso SOP	PCR thermal cycler, gel electrophoresis apparatus
Field Parameters		
Dissolved Oxygen	USEPA 360.1	YSI Multiprobe
pH	USEPA 150.1	YSI Multiprobe
Specific Conductance	SM 2510 B	YSI Multiprobe
Salinity	SM2520 B	YSI Multiprobe
Water Temperature	USEPA 170.2	YSI Multiprobe
Flow Severity	TCEQ SOP V-1	Field observation
Flow	TCEQ SOP V-1	Global Water Flow Probe
Water level	USGS	Meter stick

USEPA = Methods for Chemical Analysis of Water and Wastes, March 1983
SM = Standard Methods for Examination of Water and Wastewater, 21st edition
SOP = Standard Operating Procedure
USGS = Techniques of Water Resources Investigations, Book 3, Chapter A8, 1980

Section B5: Quality Control Requirements

Table A.7-1 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.

Field Blanks

Field blanks consist of sterile distilled water that is taken to the field and transferred to the appropriate container in precisely the same manner as a field sample during the course of a sampling event. They are used to assess contamination from field sources such as airborne materials, carryover from prior sampling sites, and containers. A field blank should be included for each sampling event. The analysis of field blanks should yield a value of no colonies detected.

Laboratory Blanks

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

Matrix spikes (MS)

A matrix spike is an aliquot of sample spiked with a known concentration of the analyte of interest. Percent recovery of the known concentration of added analyte is used to assess accuracy of the analytical process. The spiking occurs prior to sample preparation and analysis. Matrix spike samples are routinely prepared and analyzed at a rate of 10% of samples processed or one per batch whichever is greater. The MS may be spiked at a level less than or equal to the midpoint of the calibration or analysis range for each analyte. The MS is used to document the accuracy of a method due to sample matrix and not to control the analytical process. Percent Recovery (%R) is defined as 100 times the observed concentration, minus the sample concentration, divided by the true concentration of the spike. MS recoveries are indicative of matrix-specific biases and are plotted on control charts maintained by the laboratory.

Measurement performance specifications for matrix spikes are not specified in this document, and MS data should be evaluated on a case-by-case basis.

The formula used to calculate percent recovery, where %R is percent recovery; SSR is the observed spiked sample concentration; SR is the sample concentration; and, SA is the spike added; is:

%R = (SSR -SR)/SA * 100

Field Splits

A field split is a single sample subdivided by field staff immediately following collection and submitted to the lab as two separately identified samples according to procedures specified in the TCEQ SOP. Split samples are preserved, handled, shipped, and analyzed identically and are used to assess variability in all of these processes. Field splits apply to conventional samples only. According to procedures specified in the TCEQ SOP, field splits are to be submitted with every tenth sample. If less than 10 samples are collected in a month, submit one set of splits per month.

The precision of field split results is calculated by RPD using the following equation:

RPD = (X1-X2)/((X1+X2)/2)

A 20% RPD criteria will be used to screen field split results as a possible indicator of excessive variability in the sample handling and analytical system. If it is determined that elevated quantities of analyte were measured and analytical variability can be eliminated as a factor, then variability in field split results will primarily be used as a trigger for discussion with field staff to ensure samples are being handled in the field correctly. Some individual sample results may be invalidated based on the examination of all extenuating information. The information derived from field splits is generally considered to be event specific and would not normally be used to determine the validity of an entire batch; however, some batches of samples may be invalidated depending on the situation. Professional judgment during data validation will be relied upon to interpret the results and take appropriate action. The qualification (i.e., invalidation) of data will be documented on the Data Summary. Deficiencies will be addressed as specified in this section under Deficiencies, Non conformances, and Correction Action released to Quality Control.

Positive Control

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

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 Project Leader and the TSSWCB PM.

Section B6: Equipment Testing, Inspection, & Maintenance Requirements

To minimize downtime of all measurement systems, spare parts for field and laboratory equipment will be kept in the laboratory, and all field measurement and sampling equipment, in addition to all laboratory equipment, must be maintained in a working condition. All field and laboratory equipment will be tested, maintained, and inspected in accordance with manufacturer's instructions and recommendation in Standard Methods for the Examination of Water and Wastewater, 21st Edition. Maintenance and inspection logs will be kept on each piece of laboratory equipment and general maintenance checklists will be filled out for field sampling equipment, by the field technician, prior to each sampling event.

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 Project Leader and the TSSWCB PM.

Table B.6-1. Equipment Inspection and Maintenance Requirements

Equipment	Relevant Testing, Inspection and Maintenance Requirement
D.O. meter	SM 9020 B 3.a
Conductivity meter	SM 2510 B 2.c
pH meter	SM 4500-H^+ B 2 b
Thermometers	SM 9020 B 3.a
Water deionization units	SM 9020 B 3.d
Media dispensing apparatus	SM 9020 B 3.f
Autoclaves	SM 9020 B 3.h
Refrigerator	SM 9020 B 3.i
Ultra Low Freezer	SM 9020 B 3.j
Membrane filter equipment	SM 9020 B 3.k
Ultraviolet sterilization lamps	SM 9020 B 3.l
Biological safety cabinet	SM 9020 B 3.m
Incubators	SM 9020 B 3.o
Glassware and plastic ware	SM 9020 B 4.a
Utensils and containers	SM 9020 B 4.b
Dilution water bottles	SM 9020 B 4.c
Flow Meter	Product Owner's Manual
Ion Chromatograph	TCEQ SOP V-1
RiboPrinter	Per manufacturer and annual preventative maintenance
PCR thermal cycler	Per manufacturer

Section B7: Instrument Calibration and Frequency

All instruments or devices used in obtaining environmental data will be calibrated prior to use. Each instrument has a specialized procedure for calibration and a specific type of standard used to verify calibration. The instruments requiring calibration are listed below in Table B.7-1.

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 Project Leader and the TSSWCB PM.

Table B.7-1. Instrument Calibration Requirements

Equipment	Relevant Calibration Requirement
D.O. meter	SM 4500-O G 3.c
Conductivity meter	SM 2510 B 4.a
pH meter	SM 4500-H^+ B 2 b
Flow Meter	Product Owner's Manual
Ion Chromatograph (nitrates)	TCEQ SOP V-1
RiboPrinter	Manufacturer annual preventative maintenance
PCR thermal cycler	Per manufacturer's recommendations

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)

The data used to establish current bacterial loadings in the Buck Creek watershed are the result of quarterly sampling conducted through TCEQ's CRP. Buck Creek is an unclassified waterbody and as such, has not been sampled at the same quarterly intervals as the classified waterbody into which it flows. Buck Creek has been on an intermittent sampling regime as required by TCEQ's CRP in which only 14 *E. coli* samples (with 7 excedances of >394colonies per 100ml) and 20 fecal coliforms (with 12 excedances of >400 colonies per 100ml) have been collected over the course of a 5-yr period and represented only one site on a 54-mile waterbody.

Additionally, data from two other projects conducted in the Buck Creek watershed will also be utilized in this project. These data were collected under approved QAPPs and for the following projects:

1. Data collected under the *Bacterial Monitoring for the Buck Creek Watershed* project (TSSWCB Project 03-07) where taken in accordance with the approved QAPP for the project and encompasses data collected from November 1, 2003 to September 30, 2007. Data that may be used from this project include water quality, rainfall and stream flow information.
2. Data collected under the *Classification of Current Land Use/Land Cover for Certain Watersheds Where Total Maximum Daily Loads or Watershed Protection Plans Are In Development* (TSSWCB Project 08-52) where taken in accordance with the approved QAPP for the project and encompass data collected and analyzed from March 2008 to March 2009. Data that may be used from this project include global positioning points and their associated land use/land cover.

The data acquired in these projects have been collected and analyzed using similar assessment objectives, sampling techniques, laboratory protocols and data validation procedures as the current project.

Even though historical data will be considered, the data collected during the course of this project will provide the basis for a more sound scientific decision concerning the extent of the bacterial impairment of the watershed and will be taken into consideration when outlining a WPP to improve and protect the entire watershed.

Section B10: Data Management

Field Collection and Management of Routine Samples

Field staff will visit sampling sites on a monthly basis to collect grab water samples and measure field water quality parameters. Site identification, date, time, personnel, water depth, measurements of field parameters, and any comment concerning weather or conditions at the site are noted on a field data sheet. One field data sheet is filled out in the field for each site visited. An example of a field data sheet is shown in Appendix C. If no flow is observed at a site, samples will not be collected but information about the site visit will be recorded on the field data sheet and the site noted as pooled with no flow or dry. Information on the dates that sites were visited when no flow occurred will be recorded into a separate MS Excel workbook.

Field staff will measure DO, pH, water temperature, flow rate, salinity, and specific conductance at each stream site, using calibrated multi-sonde equipment. Flow rate will be recorded using a flow meter. Measurements read from the instruments will be recorded on the field data sheet. Grab samples will be collected at the site, and an identification number (either a sample identification number or a site code) will be written in permanent marker on the outside of the sterile polypropylene sample bags.

Site codes are marked on sample bags in the field. The COC forms will be used if the collecting technician is in fact not the same person receiving samples into the lab. Site name, time of collection, comments, and other pertinent data are copied from the field data sheets to the COC.

All COC and field observations data will be manually entered into an electronic database. The electronic database will be created in Microsoft Excel software on an IBM-compatible microcomputer with a Windows XP Operating System. The project database will be maintained on the computer's hard drive, which is also simultaneously saved in an external network folder. All pertinent Buck Creek data files will be backed up monthly on an external hard drive and stored in a fire proof location. Current data files will be backed up on CD-RWs weekly and stored in separate area away from the computer.

Original data recorded on paper files will be stored for at least five years in a locked, restricted-access, fire-resistant storage area. Electronic data files will be archived to CD-ROM after approximately one year, then maintained in the above storage area.

Laboratory Data

All field samples will be logged upon receipt, COC forms (if applicable) will be checked for number of samples, proper and exact I.D. number, signatures, dates, and type of analysis specified. The 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 the

Microsoft Excel based 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 Leader to ensure that there are no transcription errors. Hard copies of data will be printed and housed in the AgriLife Vernon laboratory for a period of five years. Any COC's and bacteriological records related to QA/QC of bacteriological procedures will be housed at AgriLife Vernon.

Sample Delivery to Other Laboratories

Water samples collected for nitrates analysis and fecal and water samples prepared for BST analysis will be transferred to labs outside of AgriLife Vernon.

Nitrates samples are collected and logged using the procedures described above in the field collection and lab data sections. The AgriLife Vernon 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 RRA lab. The AgriLife Vernon Research Technician, delivers the samples to RRA lab personnel at a mutually agreed upon location where they receive the samples and deliver them to the lab. COC forms are filled out accordingly until the samples reach their final destination, the RRA lab.

Water and fecal samples collected for BST analysis are collected and logged using the procedures described above in the field collection and lab data sections. The AgriLife Vernon 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 AgriLife El Paso lab. The AgriLife Vernon Research Technician ships the samples, the appropriate Sheets of Lading for Fecal Specimen Transport (Appendix D-6) and COC forms to the AgriLife El Paso lab 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 AgriLife El Paso lab, the COC forms are updated and the AgriLife Vernon Research Technician is notified of the samples receipt.

Data Reporting

Data transmission between labs (AgriLife El Paso to AgriLife Vernon, RRA to AgriLife Vernon, AgriLife Vernon to TWRI) occurs electronically. In the event that data files are too large to send via Email, a copy of the data set is copied to a CD-RW disc and mailed to the appropriate party. Data are recorded in Microsoft Excel format and submitted to the respective entity. AgriLife Vernon maintains the project database and follows the guidelines listed above in protecting the data from corruption or loss.

Data will be reported according to the standards of the TSSWCB. Data intended to be submitted by TSSWCB to TCEQ for inclusion in SWQMIS for use in 305(b) assessments will be reported

in a format consistent with *TCEQ Surface Water Quality Monitoring Data Management Reference Guide* (October 2007 is most recent version).

In reference to this, the storet for salinity and its referenced data is used only for coastal and marine waters and is not useable for inland waters, see table 3.1 on page 3-5. See the section titled Salinity on page 3-11 and Table 3-11 on page 3-29. What should be reported is specific conductance Storet 00094. I would not think you would want to submit a lot of salinity data that might not be acceptable for assessment purposes where specific conductance is the norm. My suggestion would be to submit the specific conductance for TRACs and keep salinity for office use.

Data Dissemination

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

Section C1: Assessments and Response Actions

The following table presents the types of assessments and response action for activities applicable to this QAPP.

Table C.1-1. Assessments and Response Actions

Assessment Activity	Approximate Schedule	Responsible Party	Scope	Response Requirements
Status Monitoring Oversight, etc.	Continuous	TWRI and AgriLife Vernon	Monitoring of the project status and records to ensure requirements are being fulfilled. Monitoring and review of contract laboratory performance and data quality	AgriLife Vernon and TWRI will report to TSSWCB PM via QPR.
Laboratory Inspections	Once per life of project (each lab)	TSSWCB QAO	Analytical and QC procedures employed at the laboratory	AgriLife Vernon, AgriLife El Paso and the RRA lab have 30 days to respond in writing to the TSSWCB QAO to address corrective actions
Monitoring Systems Audit	Once per life of project	TSSWCB QAO	The assessment will be tailored in accordance with objectives needed to assure compliance with the QAPP. Field sampling, handling and measurement; facility review; and data management as they relate to the project	AgriLife Vernon has 30 days to respond in writing to the TSSWCB QAO to address corrective actions

Corrective Action

The AgriLife Vernon Project Leader is 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 at AgriLife Vernon. 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.

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. AgriLife Vernon, AgriLife El Paso and RRA 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 Project Leader 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 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 such as SAS. 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.

The AgriLife Vernon Project Leader 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 D.2-1. Data Review, Verification, and Validation Procedures

Data to be Verified	Field[†] Supervisor	Laboratory Supervisor	PM/QAO Task[‡]
Collection and analysis techniques consistent with SOPs and 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
Valid STORET codes			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 for AgriLife Vernon
[‡] 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 watershed characteristics, potential sources of pollution and will aid in the selection of BMPs to address identified water quality issues. Ultimately, stakeholders will use the information produced by this project for the development of a comprehensive WPP that outlines management measures needed to address water quality concerns in the Buck Creek watershed.

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:___________________________

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CHAIN OF CUSTODY RECORD

Project: *WPP Development for Buck Creek*	**Remarks:** **AgriLife Vernon Lab to AgriLife El Paso Lab**
Name and signature of collector:	**Air bill #**

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

Relinquished by Vernon Lab Tech:	Date:	Time:	Received for AgriLife El Paso lab by:	Date:	Time:

Laboratory Notes:

Media Code: (**FW**) Fecal Isolate from Water Sample; (**FF**) Fecal isolate from Feces; (**FS**) Fecal Sample; (**SS**) Sewage Sample

CHAIN OF CUSTODY RECORD

Project: *WPP Development for Buck Creek*	**Remarks:** **AgriLife Vernon Lab to RRA-Wichita Falls**
Name and signature of collector:	**Air bill #**

Station ID	**Sample ID**	**Media Code**	**Sample Type**	**Preservative**	**Collection Date**	**Time**	**Results**
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			
		FWS	Filtered Water Sample	None			

Relinquished by Vernon Lab Tech:	Date:	Time:	Received for RRA (transport) :	Date:	Time:
Relinquished by RRA (transport):	Date:	Time:	Received for RRA Lab by:	Date:	Time:

Media Code: **FWS**- 100ml water filtered for nitrate testing

Lab notes:

Field Data Reporting Form

B | C | | | |
Station ID

Date: mm/dd/yy

Time: 24:00

Depth (inches)

B | C | | | |
Sample ID

V | C | | | | | |
COC Number

. °F
Air Temp

Collector's Last Name(s)

AgriLife Vernon

Collecting Agency

Station Description

Circle one:

Flow Severity: 1-no flow 2-low 3-normal 4-flood 5-high 6-dry

Days since last significant rainfall:

Weather Observations (use codes from back**)**

Dissolved Oxygen (DO) . mg/l

pH .

Specific Conductance , µS/cm

Water Temperature . **°C**

Flow Rate . mps

Salinity . ppt

Type of sample collected: circle appropriate sample **Water** **Fecal** **Number:** ____

Other Observations:

Weather Observation Codes:

00: No significant weather observed
01: Clouds generally dissolving or becoming less developed
02: State of sky on the whole unchanged during the past hour
03: Clouds generally forming or developing during the past hour
04: Haze, smoke, or dust in suspension in the air, visibility equal to or greater than 1km
05: Smoke
10: Mist
12: Distant lightning
18: Squalls
20: Fog during previous hour,
21: Precipitation during previous hour
22: Drizzle (not freezing) or snow grains during previous hour
23: Rain (not freezing) during previous hour
25: Freezing drizzle or freezing rain during previous hour,
26: Thunderstorm (with or without precipitation) during previous hour,
27: Blowing or drifting snow or sand
28: Blowing or drifting snow or sand, visibility equal to or greater than 1 km
29: Blowing or drifting snow or sand, visibility less than 1 km
30: Fog
31: Fog or ice fog in patches
32: Fog or ice fog, has become thinner during the past hour
33: Fog or ice fog, no appreciable change during the past hour
34: Fog or ice fog, has begun or become thicker during the past hour
35: Fog, depositing rime
40: Precipitation
41: Precipitation, slight or moderate
42: Precipitation, heavy
43: Liquid precipitation, slight or moderate
44: Liquid precipitation, heavy
45: Solid precipitation, slight or moderate
46: Solid precipitation, heavy
50: Drizzle
51: Drizzle, not freezing, slight
52: Drizzle, not freezing, moderate
53: Drizzle, not freezing, heavy
54: Drizzle, freezing, slight
55: Drizzle, freezing, moderate
56: Drizzle, freezing, heavy
57: Drizzle and rain, slight
58: Drizzle and rain, moderate or heavy
60: Rain
61: Rain, not freezing, slight
62: Rain, not freezing, moderate
63: Rain, not freezing, heavy
64: Rain, freezing, slight
65: Rain, freezing, moderate
66: Rain, freezing, heavy
67: Rain or drizzle and snow, slight
68: Rain or drizzle and snow, moderate or heavy
80: Showers or intermittent precipitation
81: Rain showers or intermittent rain, slight
82: Rain showers or intermittent rain, moderate
83: Rain showers or intermittent rain, heavy
84: Rain showers or intermittent rain, violent
90: Thunderstorm
91: Thunderstorm, slight or moderate, with no precipitation
92: Thunderstorm, slight or moderate, with rain showers and/or snow showers
93: Thunderstorm, slight or moderate, with hail
94: Thunderstorm, heavy, with no precipitation
95: Thunderstorm, heavy, with rain showers and/or snow
96: Thunderstorm, heavy, with hail

Appendix D
Standard Operating Procedures

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D-1: 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 (i.e. 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 to the Vernon lab. All fecals should be cultured within 24 hours of reaching the lab.

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.

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

D-2: Archival of *Escherichia coli* Isolates

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

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

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

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

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

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

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

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

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

D-3: 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. <u>One full PCR batch on the MJ Research Cycler 48 well-plate will have 46 samples, *E. coli* QC101, and a no template control.</u>

   <u>**ERIC-PCR Master Mix – 24 samples + 2 blanks, prepare X 2 for full 48-well plate**</u>

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

<u>Ethidium Bromide Stain (0.5 µg/mL)</u>

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)

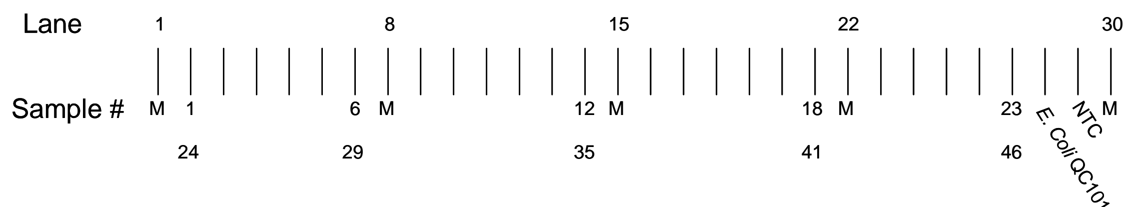
    f. Load 10 µl of sample ERIC-PCR reactions into next 6 lanes

    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.

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

D-4: RiboPrinting of *Escherichia coli*

Storing and Handling Disposables

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

Heating membrane and probe (MP) Base

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

To degas buffer:

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

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

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

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

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

Sample Preparation Procedures

1. Incubate and Inspect the Samples

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

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

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

2. Transfer Sample Buffer to Intermediate Tubes

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

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

3. Add sample buffer to microcentrifuge tubes

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

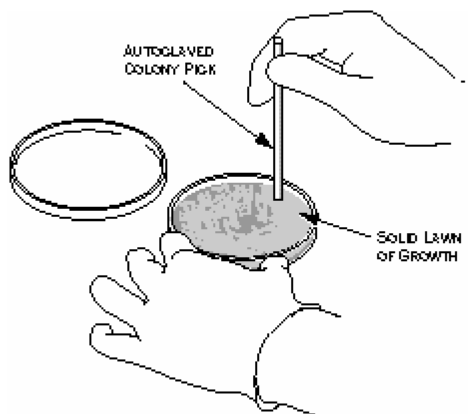
2. For Gram negative samples (including *E. coli*), add 200 µL of sample buffer from the intermediate tube.

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

3. Close the lids on the tubes.

4. Harvest the Samples

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



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

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

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

5. Mix the Samples

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

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

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

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

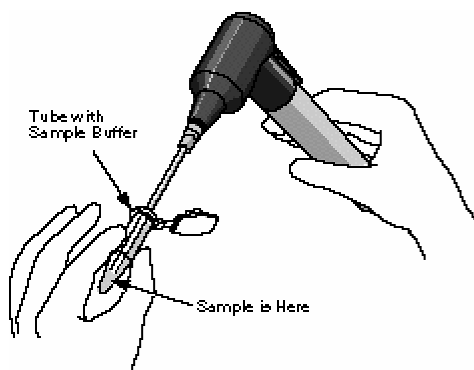
3. Press the ON lever on the mixer for about 5 seconds.

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

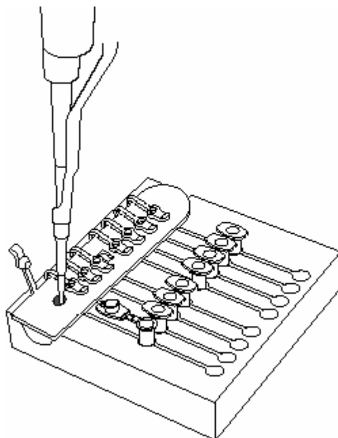
5. For **Gram positive samples only**, (e.g. *Staphylococcus* and *Listeria*) locate a new colony pick and repeat the steps for harvesting and mixing samples, adding a second sample to the original tube. Discard the used picks in a biowaste bag.

6. Cap the sample tube.

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



6. Transfer the Samples to the Sample Carrier



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

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

3. Close the lid cover for the well.

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

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

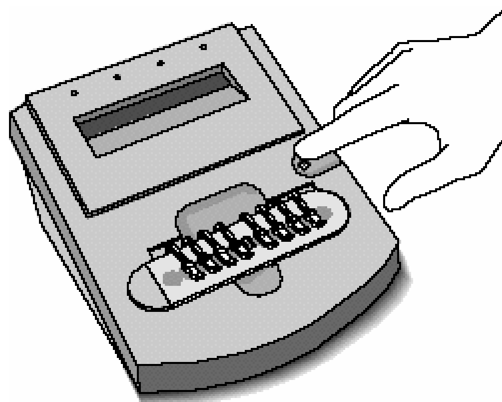
6. Lightly wipe down the outer surfaces of the sample carrier with a lab wipe wetted with surface disinfectant (10% bleach or 70% alcohol).

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

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

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

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



2. Press the button on the Heat Treatment Station.

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

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

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

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

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

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

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

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

Creating and Loading a Batch

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

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

You can also create special batches:

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

From the Instrument Control Base Window:

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

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

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

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

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

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

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

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

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

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

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

10. Repeat for the other seven samples.

11. Click on Save and Submit Batch to Instrument.

Loading Disposables

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

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

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

1. Check the DNA Preparation Waste Container

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

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

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

2. Load the Sample Carrier

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

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

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

3. Load the DNA Prep Carrier

1. Remove the DNA Prep carrier from the refrigerator.

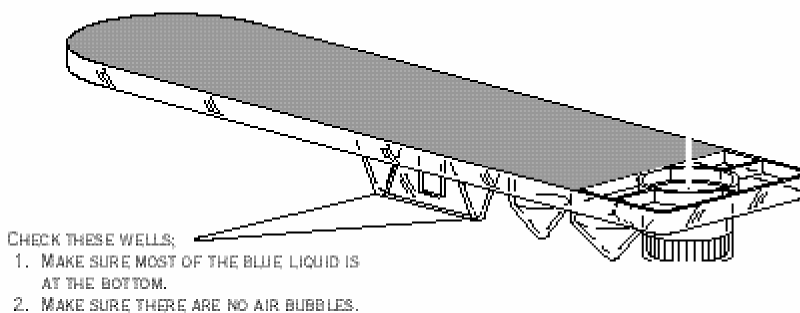
2. Check the wells in the carrier. If most of the liquid appears to be in the bottom of the wells and there are no bubbles, go to step 3. Otherwise **lightly tap the side of the carrier a few times with your finger to release any material that has adhered to the lid.**

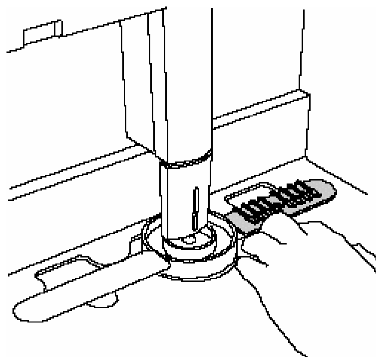
3. CAUTION! Do not tap the carrier briskly. This may cause the marker to degrade which can create inaccurate results.

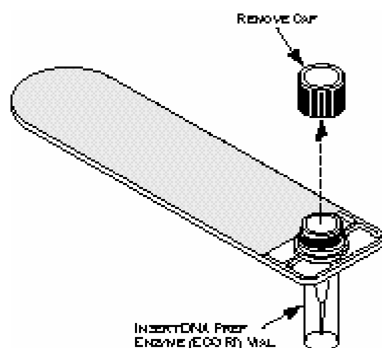
4. Remove a vial of DNA Prep Enzyme (*Hin*d III or *Eco*R I) from the freezer. ***Hin*d III (NEB Cat. #R0104M) is prepared in a Sarstedt 500-µL microfuge tube (Cat. #72730-005) as a 50 U/µL working stock as follows.**

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

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





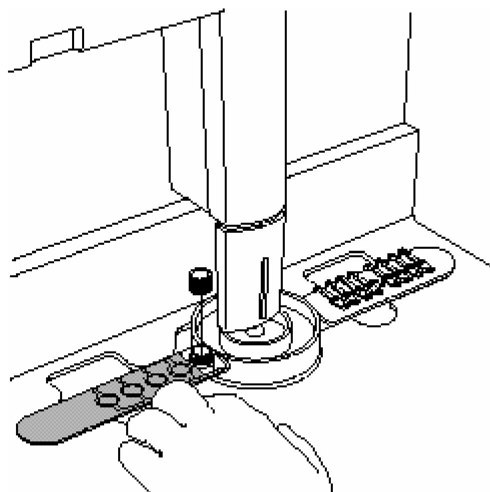


5. Remove the cap from the Enzyme vial.

6. Insert the vial into the carrier.

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

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

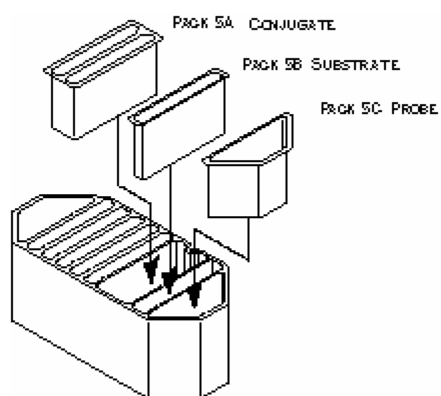


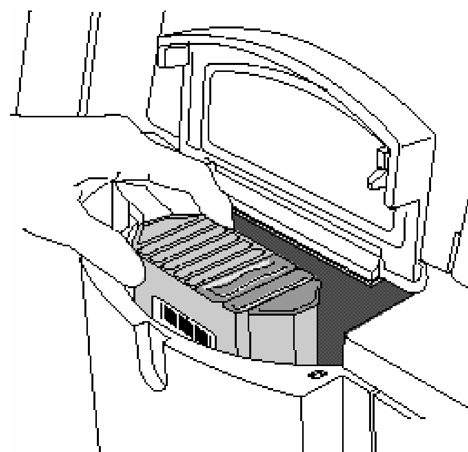
4. Load the MP Base and Carousel

1. Unpack the disposables.

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

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





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

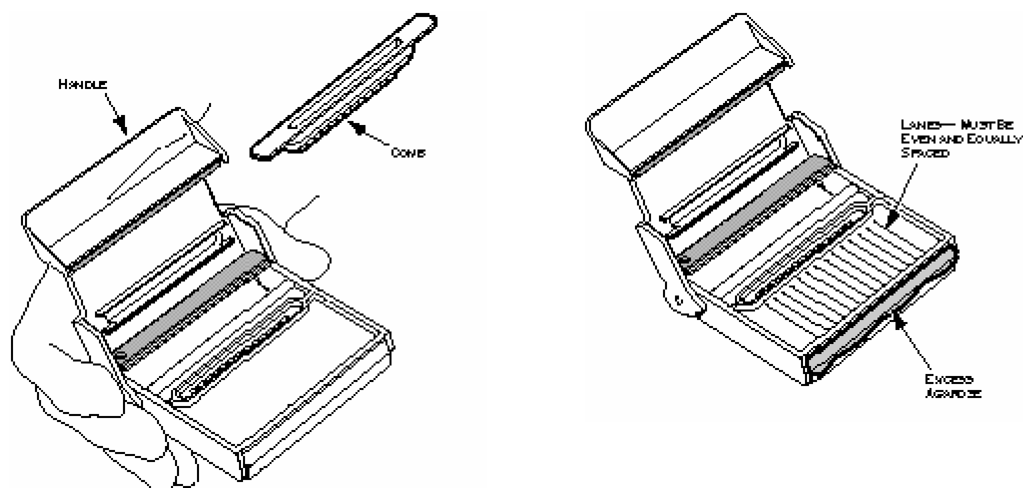
5. Load the Gel Cassette

1. Remove the gel cassette from its package.

2. Grasp one end of the rubber comb and gently pull the comb from the cassette.

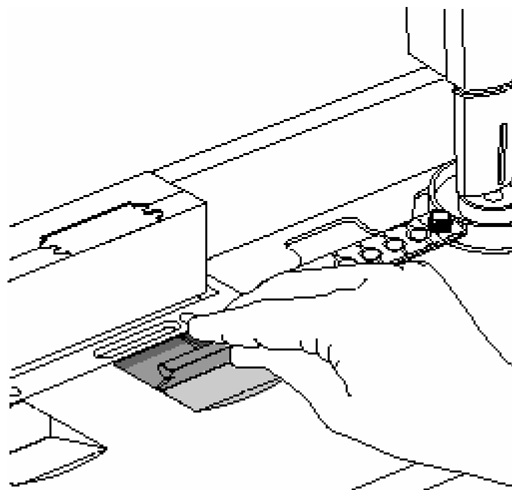
3. Unfold the handle of the cassette towards you until the handle snaps into place.

4. Check the front edge of the gel cassette and the lanes of the gel.



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

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

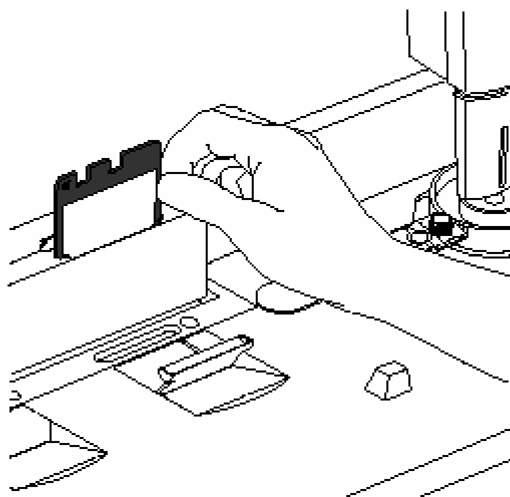


6. Press the cassette forward firmly until it snaps into place.

6. Load the Membrane

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

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



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

8. Load the Next Batch

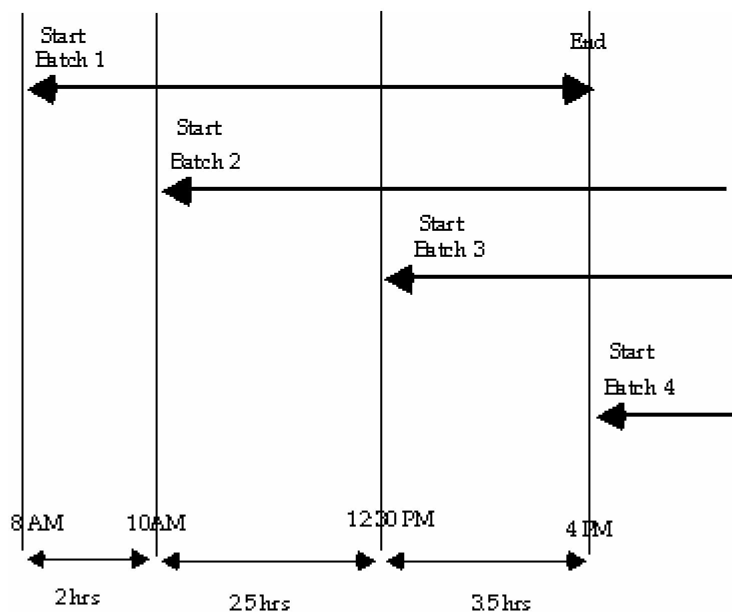
The RiboPrinter® microbial characterization system lets you load up to four VCA batches in an eight hour period. Other batches may take longer to process.
The chart above shows the approximate loading times for each batch in a work shift using only the VCA protocol.

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

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

Batch Report

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



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

D-5: *Bacteroidales* PCR

Preprocessing of Water Samples
(AgriLife Vernon)

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

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

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

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

4. Ship frozen filters on dry ice in insulated coolers by next day courier to:

   Dr. George D. Di Giovanni
   Texas AgriLife Research and Extension Center
   1380 A&M Circle
   El Paso, TX 79927
   915-859-9111

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

DNA Extraction and PCR
(AgriLife El Paso)

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

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

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

4. Transfer lysate to a 2.0 ml microfuge tube.

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

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

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

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

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

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

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

Bacteroidales PCR Master Mix

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

Bacteroidales PCR Master Mix – per sample

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

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

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

4. Carefully seal plate using an adhesive PCR cover.

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

    a. UDG digestion 50°C for 10 min

    b. Initial denaturation at 95°C for 10 min

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

    d. Final Extension at 72°C for 10 min

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

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

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

9. Allow gel to solidify for approximately 30 to 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 Logic 200 SOP for image capture. Save digital photograph as an 8-bit TIFF file with no scaling and print a hardcopy for notebook.

D-6: Sheets of Lading for Fecal Specimen Transport

Texas AgriLife Research and Extension Center
Vernon, Texas
Buck Creek Project
Phyllis Dyer, Research Tech II
(940)552-9941x247

In case of EMERGENCY:
John Sij Phone: (940)552-9941x232

Date:____________Time:______________

Sample: Fecal **Hazard:** Bacteria

Species:______________________________

Photo: **Yes** **No**

GPS: Lat______________Long____________

Technician:___________________________