The California Microbial Source Identification Manual:
A Tiered Approach to Identifying
Fecal Pollution Sources to Beaches

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DISCLOSURE

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LIST OF ACRONYMS

CA: California

CBI: Clean Beaches Initiative

CCTV: Closed Circuit Television

CIWQS: California Integrated Water Quality System

DNA: Deoxyribonucleic Acid

ELISA: Enzyme-linked Immunosorbent Assay

ENT: *Enterococcus*

EPA: Environmental Protection Agency

FIB: Fecal Indicator Bacteria

GIS: Geographic Information Systems

GPS: Global Positioning System

MCA: Microbial Community Analysis

MST: Microbial Source Tracking

OB: Optical Brighteners

OWTS: Onsite Wastewater Treatment System

PCR: Polymerase Chain Reaction

POTW: Publicly Owned Treatment Works

qPCR: Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

RV: Recreational Vehicle

SIPP: Source Identification Protocol Project

SOP: Standard Operating Procedure

SSMP: Sewer System Management Plan

SSO: Sanitary Sewer Overflow

TMDL: Total Maximum Daily Load

TRFLP: Terminal Restriction Fragment Length Polymorphism

WDR: Waste Discharge Requirements

CHAPTER 1: INTRODUCTION

California's beaches represent an important recreational and economic resource. Almost 300,000 jobs relate to coastal tourism and recreation, accounting for 75% of employment in the state's ocean economy sector and contributing $12 billion to the state's gross domestic product. Fecal pollution at beaches poses both aesthetic and health concerns for visitors. Beach advisories or closures due to fecal contamination detection may result in local economic losses of up to $100,000 per day.

Recognizing the value of beaches to California residents and the state's economy, the California Clean Beaches Initiative (CBI) Program has provided approximately $100 million since 2001 for projects to improve beach water quality at many of the state's most contaminated beaches. These funds have gone toward implementing best management practices, such as diverting storm drains to reduce runoff flows during the dry season, repairing aging sewer lines, and creating natural runoff filtration areas.

Despite the CBI's successes, a number of beaches still frequently exceed water quality standards for fecal indicator bacteria (FIB) and lack cleanup strategies, largely because the fecal contamination sources are unknown. FIB can originate from numerous sources, including human sewage; manure from livestock operations, wildlife, or urban runoff; and even growth in the environment. In any given water body, effective management and mitigation requires knowledge of the source(s) of fecal contamination and of FIB, which are not always the same.

Microbial source tracking (MST) methods can identify FIB host sources, including those that carry the greatest human health risk. Some new MST methods discriminate between human and non-human fecal sources, and others distinguish among several animal sources. However, these methods do not perform equally well. Within the plethora of methods, each has a different set of advantages. To improve understanding of method performance, the CBI funded a comprehensive MST method evaluation study in 2011, referred to as the Source Identification Protocol Project (SIPP). The SIPP quantified specificity and sensitivity for 41 MST methods.

Still, many questions remain: how to employ different marker types and how new genetic source identification methods might be combined with traditional methods, such as dye testing of pipe networks. Thus, agencies charged with identifying FIB sources to beaches may hesitate to move forward with studies or, conversely, deploy too many techniques, which can be prohibitively expensive.

To address these information gaps, the CBI supported the SIPP in developing this source identification manual. It provides guidance for cost-effectively identifying sources of fecal contamination within a watershed. The manual is based on a hypothesis-driven and tiered approach, in which the user implements the least expensive options first and more expensive tools only when sufficient uncertainty warrants their use.

1.1 The Tiered Approach

This document describes six tiered steps to implement a hypothesis-driven, science-based microbial source identification approach, while conserving resources through progressive deployment:

1) Characterize the watershed by obtaining infrastructure maps, interviewing relevant local experts, and conducting visual inspections to develop a list of potential fecal contamination sources.

2) Examine historical and current FIB monitoring data for spatial and temporal trends to help identify conditions that result in elevated FIB levels and examine linkages to the greatest potential sources of fecal contamination.

3) Where leakage from a sanitary system is a potential source, investigate it using traditional tools such as smoke testing, dye testing, or camera inspection.

4) Where human sources are a potential contributor, test ambient waters for human source-specific genetic markers (even if traditional tools have not identified a leaking sanitary system). Place high priority on either detecting or confirming a human fecal source, as this source may pose the greatest relative health risk.

5) Where human sources have been accounted for and the relative human loadings are better understood, and/or a likely animal fecal pollution source (e.g., runoff from a horse farm) has been identified, test ambient waters using non-human (animal) source-specific genetic markers.

6) Where source-specific genetic markers have yet to be developed for the suspected source(s), consider testing ambient waters using genetic community analysis methods. Users may also be able to employ these methods to differentiate sources within common host types (e.g., human fecal pollution from sewage vs. septage). These methods may be complemented with chemical methods (as appropriate) to provide additional independent information, particularly when sewage or other human waste is a suspected source.

The chapters in this manual provide procedures or design guidance to implement each of these steps. In addition, pointers reference other documents where guidance already exists for that activity, and case studies (shown in boxes) illustrate successful implementation examples. Finally, the appendices detail standard operating procedures for each of the manual's recommended genetic test methods. This manual provides guidance to agencies charged with identifying FIB sources to beaches. For additional information or questions, please contact Dr. John Griffith (johng@sccwrp.org).

CHAPTER 2: (STEP 1) GATHERING INFORMATION TO FORMULATE HYPOTHESES ABOUT POTENTIAL FECAL SOURCES

Source identification begins with gathering information to develop hypotheses about potential fecal contamination sources. This step sets the stage for cost-effective, targeted sampling to either confirm or de-emphasize hypothesized sources using appropriate investigative techniques.

Hypothesis generation involves five main activities:

- Meet with local experts who have knowledge of the watershed to obtain and synthesize relevant information about possible sources;
- Gather information about the location, age, and condition of local storm and sanitary sewer infrastructure;
- Gather historical FIB monitoring data along with monitoring location maps;
- Conduct field reconnaissance by walking the beach and watershed to visually identify possible sources;
- Synthesize information to generate hypotheses.

2.1 Meeting with Stakeholders

Local organizations with institutional knowledge about the watershed, beach, and infrastructure often hold the best available information about potential bacterial contamination sources. The first step is to identify these potential collaborative organizations and initiate information transfer via phone, in-person interviews, and/or stakeholder meetings. In watersheds with on-site wastewater treatment systems, also seek to engage property owners through community education/outreach and public meetings. Potential collaborative organizations include (but are not limited to):

- Municipal agencies responsible for maintenance of stormwater and/or sewage systems,
- Environmental health agencies who are routinely monitoring beach and tributary surface water quality for FIB,
- Wastewater treatment agencies,
- California State Parks,
- The California Coastal Commission,
- Regional Water Quality Control Board(s),
- Local military installations,
- Local universities and other research institutions that study water quality in the area, and
- Local nongovernmental organizations that conduct citizen monitoring.

In many cases, responsibility for sanitary and stormwater infrastructure upkeep spans multiple agencies and jurisdictions throughout the watershed associated with the beach. For example, cities may maintain smaller sanitary and stormwater collection systems, while the county may monitor beach water quality and manage the main storm drain channels. Likewise, municipalities

may own and maintain the sanitary collection system within their borders, but contract out wastewater treatment to a sanitation district. Anyone who has knowledge of the water infrastructure, including monitoring data, should participate in the information reconnaissance step.

At the first stakeholder meeting, present historical data and discuss it with participants, focusing on how they can provide missing information. Request from regulatory agencies (Regional Water Quality Control Boards) any available information and data associated with Total Maximum Daily Loads (TMDLs), prohibitions, Sanitary Sewer Overflow (SSO) Reduction Programs, or other programs that require monitoring and reporting. Ask the municipal agencies responsible for the sanitary and stormwater infrastructure to provide any data necessary to complete GIS maps. In addition, much valuable information to inform source investigation already resides in their Sewer System Management Plans (SSMPs), which are prepared in accordance with state regulations (*see Box 2.1*). Look for additional information in documents related to Illicit Connections/Illicit Discharges (ICID) regulations.

Note: The initial stakeholder meeting represents the first conversation of an ongoing dialogue between stakeholder group members and the study team. If you later identify additional stakeholders, include them in future meetings.

Box 2.1: Documents available through regulatory requirements

In California, the State Water Resources Control Board adopted Statewide General Waste Discharge Requirements (WDRs) for Sanitary Sewer Systems via Water Quality Order No. 2006-0003 on May 2, 2006. The State updated the requirements in 2008 and anticipates additional updates in 2013. Visit http://www.waterboards.ca.gov/water_issues/programs/sso/index.shtml to find documentation. The WDRs apply to public agencies that own or operate sanitary sewer systems greater than one mile long. They require these agencies to develop and implement sewer system management plans (SSMPs) and report all sewer system overflows to an online database. The SSMPs must contain an up-to-date map of the collection system, a condition assessment and rehabilitation plan to identify and prioritize system deficiencies needing correction, and a schedule for addressing deficiencies. Other areas relevant to source identification efforts include operation and maintenance descriptions, performance goals, the overflow emergency response plan, the fats/oils/grease control program, the capital improvement plan, and the public communication program. Note any missing, incomplete, or inadequate documents as well as patterns of sewage spills.

2.2 Mapping Topographic, Infrastructure, and Land Use Information

Leaking sanitation infrastructure represents a high-risk fecal contamination source, and you should gather information about the location and condition of this infrastructure early in the investigative process. Include the location of septic tanks/leach fields, pump stations, collection systems, and publicly owned treatment works (POTWs) or privately operated wastewater treatment facillities. Identify the waste disposal system for all land parcels near the beach. Note portions of the system with deferred maintenance and frequently reported SSOs to help identify areas most susceptible to sanitary sewer failures. Also, check for areas where lines have been recently replaced or slip-lined to avoid wasting effort in locations where problems were previously identified and repaired.

If this resource is available, enter this information into a GIS mapping system, which allows synthesis of the many different data types related to the study site. With GIS layers, you can spatially examine relationships between storm and sanitary infrastructure, geographic features, land use patterns, and population density. GIS data usually already exist for stormwater and sewer infrastructure managed by public agencies. If the watershed crosses multiple jurisdictions, integrate individual GIS shape files from each jurisdiction in order to create a comprehensive map of all infrastructure systems potentially affecting the beach.

Areas with privately owned Onsite Wastewater Treatment Systems (OWTSs) may not have readily available GIS data; however, some publicly available data may exist in the local county permitting or planning agency's real estate records. Inspection records should indicate the location of both tanks and leach fields. You should build a GIS layer with the approximate latitude and longitude coordinates obtained from these records if available. Additional information on the location and condition of OWTSs may be available through documents related to the AB885 regulations.

Map land use information to identify activities in the watershed that may contribute FIB to the beach. Layers should include residential areas, crop farming, livestock operations, horse stables, and dog parks. Depending on the watershed's data availability, additional valuable layers might include features such as piers, golf courses, or homeless encampments. If possible, note locations with landfills, biosolid dispersal, or recycled water irrigation.

Add to the database the spatial locations of FIB monitoring in the watershed, based on local agency monitoring data (Chapter 3). If possible, categorize data into magnitudes for differential display on the map. Co-displaying FIB data (especially data gathered over a long period) within maps of infrastructure and key watershed features can be quite useful in forming hypotheses regarding fecal sources to creeks, streams, and the beach.

2.3 Conducting Field Reconnaissance to Identify Visible Sources

Thorough watershed reconnaissance usually means walking the area. This typically starts at the beach and moves up along the main stem of the watershed, branching out into smaller drainages as needed to identify all potential point and non-point fecal contamination sources.

Sanitary Surveys

You may choose to walk the watershed informally or as part of a formal sanitary survey. Sanitary surveys standardize collection of both physical and observational data. Physical parameters include water temperature, wave height, alongshore current direction, wind speed, and precipitation. Observational data include the number of swimmers, types and numbers of wild and domestic animals, flows from storm drains or creeks, tidal conditions, and presence of beach debris. This information may help explain both short-term water quality changes and correlative relationships between beach conditions and FIB concentrations. Aim to conduct sanitary surveys several times throughout the year to capture seasonal variations in the above parameters.

To aid in assessing the beach, the Environmental Protection Agency (EPA) has developed detailed guidance on how to conduct a sanitary survey, including useful forms and checklists for

conducting study site reconnaissance. Download the EPA Marine Beach Sanitary Survey User Manual at http://water.epa.gov/type/oceb/beaches/upload/Marine-Beach-Sanitary-Survey-User-Manual-March-2013.pdf. Check with local monitoring agencies for available data, as many agencies gather the data needed to complete sanitary surveys as part via their normal routine water sample collection procedures.

The EPA manual describes two different types of surveys: A "Routine On-Site Sanitary Survey" and an "Annual Marine Beach Sanitary Survey." If local monitoring agencies have adopted the EPA's routine survey form, data needed to complete the annual survey form will be directly transferable. The routine survey involves a simple two-page form intended to record physical and observational data as each water sample is collected. The annual survey uses a 13-page form designed to facilitate a comprehensive watershed assessment. It both inventories information to be collected when characterizing a site and contains a tool to synthesize daily observations and examine water quality trends as they relate to other parameters.

Infrastructure Sources

Starting from the beach, note the location of all drainages flowing into the main stem of each storm drain or channel flowing to the beach. Photograph each flowing sub-drain and record its GPS coordinates to validate against your GIS map. When possible, identify the source of the flow. Be careful not to overlook any flowing drains, since even a small volume of water with very high FIB concentrations can contaminate a much larger volume of water. Repeat watershed reconnaissance several times, as many drains flow intermittently and you may miss a potentially large source of bacteria if limited to a single exercise. Also, note the location of storm water diversions (if present) as well as their size and capacity.

While in the field, record the GPS coordinates of each identified onsite wastewater treatment system (OWTS). You may need to work with the county or other local agencies to obtain access to private land adjacent to a water body. Note the proximity of leach fields to flowing surface water. Look for ponding water, signs of sogginess, and vegetation indicating saturation in the areas where leach fields are expected. Also, look for large trees near tanks and leach fields, which can disrupt the system with their roots. Notice any water seeping from the sides of the creek bed near these systems.

Non-Point Sources

During field reconnaissance, keep an eye out for non-point sources where fecal material may directly enter or wash into the drainage system. Fecal material from individual humans is capable of spreading disease, so be sure to visually inspect the watershed for evidence of homeless encampments. Pay particular attention to areas under bridges and brushy areas alongside and in creek beds.

When an encampment is located, inspect the surrounding area for evidence of cesspits or human excrement that may affect water quality. The nearest flowing water may be used to carry away waste from homeless encampments. Inspect areas such as large drain openings, creek banks, and swales. If homeless persons are living in recreational vehicles or automobiles, check for sewage discharge or leaking from the RV's sanitary system or manual emptying of "chamber pots" onto the street or into nearby storm drains.

Other notable non-point sources include roaming domestic and wild animals. Flocks of birds passing through tidal estuaries or deer and raccoons in riparian habitats, for instance, can produce significant amounts of FIB. Note areas frequented by domestic or stray dogs. Likewise, check for pet waste in residential areas where drainages border homes. Record any evidence of excrement found in or near creeks and drains.

In rural or semi-rural agricultural areas, inspect livestock operations and horse stables to determine how the operator disposes of manure. Manure often ends up in a culvert or creek. Note where BMPs are preventing direct deposition of livestock feces into water bodies, as microbial attenuation from fecal aging can significantly lower the potential risk of health impacts compared to direct deposition. For crop-farming operations, check to see if runoff from fields reaches the drainage. Also, note portable toilets used by field laborers or others, including the frequency of pump-outs and the responsible parties.

2.4 Synthesizing Gathered Information to Generate Hypotheses

Information gathered during beach and watershed reconnaissance, meetings with local experts, evaluating the historical monitoring data for its spatial patterns, and GIS mapping should form the basis for generating hypotheses as to the likely sources of FIB to the beach. The objective of this step is to identify a wide array of possible sources to systematically evaluate in later tiers of the source identification process.

A hypothesis is simply a statement that one can evaluate as true or false. Each statement proposes an explanation for the observed phenomena, in this case the bacterial water quality problem at the beach in question. For example, one testable hypothesis states, "The sanitary sewer system is leaking and raw sewage is being released into the ocean." An example of a poor (untestable) hypothesis might be "All of the FIB come from swimmers." In this statement, it is not possible to definitively evaluate whether "all" of the FIB come from swimmers. An improved version of this hypothesis would be "Swimmers are a major contributor to FIB at the beach."

In generating hypotheses, consider the possibility of faulty sanitary sewer infrastructure as a high priority, since this source carries high disease potential, can be easily investigated, and typically has a straightforward solution. Sanitary sewers near beaches are often aged and may be made of materials, such as cast iron, that are subject to corrosion when exposed to salt water. In addition, shifting sands, tree roots, or seismic activity commonly disrupt the gravity portion of these collection systems. GIS map examination can help to determine if a sanitary sewer is in close proximity to the beach. Special attention should be paid to pump stations and locations where sanitary and storm sewer lines run in parallel or cross, as these are the most likely points where sewage leaking from sanitary lines may enter the storm drain system.

Septic systems also carry high disease potential and can contribute FIB to adjacent surface water if the system is failing, overloaded or simply located too close to surface waters. Consider the age and depth of the system when developing hypotheses related to OWTSs. Even a properly functioning septic system, combined with a high water table, can contaminate the groundwater that, in turn, exchanges with surface and marine waters near the coast. Many problems that affect sanitary sewer conveyance systems also affect septic systems, such as corrosion from saltwater

or disruption by tree roots. Pay special attention to any parcels where the leach field is obviously failing (indicated by saturated soil, ponding water, etc.). California's AB885 regulations provide formal definitions of a failed septic system.[1]

Another common source of FIB to beaches are conveyances for stormwater, which in the case of many streams, creeks, rivers, and storm drains may discharge near the top of the beach and run over the sand into the water. During dry weather, storm sewers may carry FIB-containing groundwater and nuisance runoff to the beach. They may also convey contaminated water from sources such as illicit connections or leaking sanitary sewers farther up the watershed. Further, tidal flushing of storm drains has been known to introduce FIB from bird droppings and decaying plant material into beach water.

Geographic features such as piers often relate to FIB contamination and leaking sanitary sewer infrastructure under piers has been observed in the past. Other human activities that generate FIB near piers, such as cleaning fish, have been well documented. Further, birds roost on piers and their droppings may be deposited directly into the water or washed off the pier by maintenance workers. The continuous shade under piers may contribute to higher levels of FIB in sand due to the lack of solar inactivation. Also take note of good management practices, such as fish cleaning stations tied into the sanitary sewer, that may help narrow the list of potential sources to be investigated.

Finally, land-use patterns can greatly affect bacterial concentrations in water. Each land use has the potential to contribute different sources of FIB. For example, urban areas may contain bacteria from leaking trash dumpsters or restaurants that hose food waste into storm drains. Agricultural operations rely on fertilizer application and may not have adequate restroom facilities for field workers. Runoff from agricultural land containing fertilizers, which help FIB to grow in the environment, or human waste deposited in fields and washed into streams by irrigation water are well-documented sources of FIB.

Sound hypotheses serve as the basis for a study design, and are essential to a successful source identification effort. Once you have generated a list of reasonable, testable hypotheses, use the methods described in Chapters 3 through 7 to evaluate them.

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[1] The full text of the State Water Resources Control Board's OTWS policy, including definitions of system failure, can be found at http://www.swrcb.ca.gov/water_issues/programs/owts/docs/owts_policy.pdf.

CHAPTER 3: (STEP 2) USING FECAL INDICATOR BACTERIA DATA TO EVALUATE HYPOTHESES AND PRIORITIZE SOURCES FOR FURTHER INVESTIGATION

The second source identification step uses cultivable FIB, such as *Escherichia coli* and *Enterococcus* to test and prioritize hypotheses generated in the first tier. This initial evaluation tier uses FIB data because they often already exist from routine monitoring. Where FIB data do not already exist, they cost less to collect compared to other fecal contamination identification methods; in addition, local agencies or volunteer groups can typically collect FIB data without a high level of expertise or specialized equipment.

Evaluating source identification hypotheses begins with examining spatial and/or temporal patterns in FIB data. Temporal patterns examine whether high FIB concentrations on the beach are associated with phenomena such as tides or precipitation. Spatial patterns elicit whether certain portions of the watershed see an increase in FIB concentrations, such as confluence with a tributary or features such as piers or bird colonies. The sections below elaborate on initial hypothesis testing using FIB data; more expensive confirmatory technology will focus only on the most likely sources.

3.1 Gathering FIB Data

You may need to collect additional FIB data to evaluate alternative hypotheses. In particular, examining spatial patterns in the watershed may require samples from a larger geographical area than those collected in routine monitoring programs. Data to examine long period temporal patterns at the beach, in contrast, are often available from historic sources. Sources include a) Heal the Bay (http://brc.healthebay.org) in Southern California and b) the California Environmental Data Exchange Network (http://www.ceden.us/AdvancedQueryTool), which compiles monitoring data from all available sources statewide, including local health departments and monitoring programs required by discharge permits. In some watersheds, additional useful FIB data from special studies or from citizen monitoring groups may be available for locations that are not routinely monitored.

Additional monitoring requirements may apply to some beaches and associated watersheds, such as those associated with FIB-based Total Maximum Daily Loads (TMDLs). Freshwater bacterial TMDLs sometimes require stormwater agencies to conduct monitoring at multiple locations throughout the watershed. Find site-specific information on current TMDLs and Regional Board prohibitions at the "Safe to Swim" website administered by the California Water Monitoring Council (http://www.mywaterquality.ca.gov/safe_to_swim/index.shtml). Sanitary sewer overflow (SSO) monitoring data appear in the California Integrated Water Quality System (CIWQS) database at http://www.waterboards.ca.gov/water_issues/programs/sso.

3.2 Examining Spatial FIB Patterns

Most commonly, evaluating your hypotheses will require examination of spatial FIB patterns. For instance, does FIB concentration increase after the confluence of each major tributary? Alternatively, do FIB concentrations change as the characteristics of the watershed change, such as from agricultural to urban land use? Assess spatial trends in dry and wet season data separately, or use a monthly average. Geometric means may be superior to averages depending

on the distribution of data at a particular site. In examining these spatial patterns, consider not only FIB levels, but also FIB loading from identified fecal sources. Even with a high FIB level in the upper watershed, the small water volume reaching the beach may not be meaningful when diluted by the larger volumes in the lower watershed, allowing its elimination as a high-priority source.

Assess whether contamination is spatially associated with specific features on the beach, such as piers or storm drains. When examining the spatial relationship of a feature to FIB, take into account the prevailing current direction when the sample was taken. You can infer current direction from measured field data or ocean buoy data on wind and swell direction in the vicinity of the study site.

Finally, pair FIB data with GIS maps of existing infrastructure. Examine GIS maps of sanitary sewers, storm sewers, and OWTSs overlain with FIB data to look for relationships between persistent FIB contamination, high levels of FIB contamination, and proximity to infrastructure. Take into consideration the age and general condition of infrastructure as well as evidence of infrastructure degradation from SSMPs, such as the frequency of leaks that have required repair, to gauge the general condition of the system and the likelihood of additional leaks. A spatial relationship between proximity to sanitary sewer infrastructure or OWTSs and increased FIB in field data suggests the sanitation system(s) should be investigated as a potential source. When you suspect faulty infrastructure, bring this to the attention of the stakeholder group. Often engineers, field workers, and health agencies have institutional knowledge about events such as sewage spills or emergency repairs to pipes that may not have been memorialized in routine maintenance records.

Box 3.1. Using spatial patterns to eliminate a potential source

Avalon Beach on Santa Catalina Island, CA shares a harbor with hundreds of moored boats, a potential fecal pollution source. Researchers carried out a short-term survey to determine if the boats contribute to the beach's pollution problems. The study aimed to determine if contamination is pervasive throughout the harbor (including sites adjacent to boats), or restricted to the shoreline. The harbor was sampled during both a rising and falling tide, assuming the net flow of water would be into the bay during the rising tide, and out of the bay during the falling tide. Sampling was conducted at night because sunlight reduces contamination levels during the day, obscuring spatial patterns that may be useful for inferring sources. Investigators found that enterococci and *E. coli* concentrations were highest at the shoreline and low within the bay, refuting the idea that boats are a major source of contamination. This information, along with a long-term study of groundwater quality and molecular source tracking marker data, led the researchers to conclude that groundwater contaminated by leaky sewage infrastructure was polluting the beach.

For additional information see: Boehm, A.B., Fuhrman, J.A., Mrse, R.D. and Grant, S.B. 2003. Tiered approach for identification of a human fecal pollution source at a recreational beach: case study at Avalon Bay, Catalina Island, California. Environmental Science and Technology 37(4), 673-680.

> **Box 3.2. Using a mass balance approach to eliminate a potential source**
>
> At Cowell Beach in Santa Cruz, CA, water flowing from a storm drain near the stairs at the north end of the beach was a suspected FIB source. This drain was measured weekly for FIB and flow rate during the 2011 and 2012 summer seasons. The mean FIB concentrations in the drain were 2400 MPN/100 ml (EC) and 2000 MPN /100 ml (ENT), much higher than the mean concentrations at the beach of 630 MPN/100 ml (EC) and 230 MPN/100 ml (ENT). The water discharged from the drain at a very low rate (~40 L/min). Given the distance between the drain and the beach (400 m), and the dilution likely to take place once the drain water entered the ocean, researchers calculated that this was not likely a main source of pollution. This conclusion was further supported by salinity data collected along with each Cowell Beach sample. On average samples contained ~1% freshwater in surf zone samples, suggesting that the freshwater storm drain source could not be responsible for the FIB at the beach.[1]
>
> *For additional information see: Russell, T.L., Sassoubre, L.M., Wang, D., Masuda, S., Chen, H., Soetjipto, C., Hassaballah, A. and Boehm, A.B. 2013. A coupled modeling and molecular biology approach to microbial source tracking at Cowell Beach, Santa Cruz, CA, United States. Environmental Science and Technology dx.doi.org/10.1021/es402303w.*

3.3 Examining Temporal FIB Patterns

In many cases, FIB contamination may be temporally associated with predictable natural phenomena, such as precipitation, tides, or seasons. First, evaluate whether the FIB problem is associated with storm or non-storm creek flows or occurs year-round. If there is a lagoon, information on when the lagoon breaches can be critical for assessing the role of the lagoon; lifeguards may record data about the berm status. Rainfall and the resulting stormwater discharge is a well-documented FIB source at California beaches and should be the first temporal variable examined. Compare the time series of FIB and precipitation to see if peak FIB counts follow precipitation events. Confirm this relationship with statistics, for example, a 2x2 contingency table test of presence of rainfall in the last three days versus FIB criteria exceedance (both binary variables).

Second, examine seasonality. While seasonality could be associated with weather patterns and rain, it might also be tied to specific human or animal activities in the watershed. For instance, in watersheds with heavy farming activity, consider whether increases in FIB concentration relate to periods of high fertilizer use. Similarly, some watersheds experience seasonal use for fairgrounds or horse racing venues, and temporal FIB patterns can elicit whether these are likely sources. In addition, bird populations at some beaches fluctuate seasonally due to migration, and FIB levels may be correlated to the number of birds.

Another seasonal factor to consider is whether or not storm drains or creeks were flowing or dry on the day the beach was sampled. Many creeks, especially in Southern California, develop a sand berm that intermittently blocks the flow of water to the beach, sometimes for many months. In addition, storm drains may be physically diverted to the sanitary sewer system during certain parts of the year. Statistical tests can help determine whether FIB levels differ among months or by other seasonal conditions, such as open-berm versus closed-berm periods.

Third, look for temporal patterns linked to tides. Tidal trends in beach water quality reflect the importance of both groundwater and beach-based FIB sources. In addition to increased surface water (rivers and creeks) influence at low tide, submarine groundwater discharge (i.e., groundwater discharged from a coastal aquifer to the ocean) is typically greater during low tide. Higher FIB during low tides may implicate groundwater as a primary source if there are no nearby rivers, creeks, or lagoons with discharge modulated by the tide. Beach-based sources, such as contaminated sand or FIB-laden beach wrack, would be more influential during high tide when most of the beach is inundated with seawater. Thus, higher FIB during high tides may tell you to look more closely at sand and wrack as important sources. In addition, spring tides bring higher than normal high tides, and lower than normal low tides that can flush out material from estuaries and creeks where these sources are present. Performing a correlation analysis with FIB concentrations and tide height or tide range will reveal whether and how FIB are associated with tides. Other tests can discern differences between FIB counts under categorical tide conditions (e.g., rising/falling or spring/neap tides; see Box 3.3).

Fourth, examine correlation with weather conditions, specifically strong winds and high surf. Higher FIB concentrations under high-energy conditions may indicate remobilization of FIB reservoirs in beach or nearshore areas. Similar correlative statistics as described above for rainfall and tide can be used for these and other weather variables.

A fifth pattern to examine is diurnal (time-of-day) trends, which can indicate the possibility of direct inputs from bathers, whose numbers vary throughout the course of the day. Diurnal patterns can also provide information on the potential importance of sunlight in controlling FIB concentrations, indicating whether sunlight should be controlled for during any remaining sample collection. If so, additional samples should be collected before dawn and then throughout the course of the day along with measurements of solar radiation.

A sixth factor to consider is near-shore ocean currents. Current velocity and direction can be modeled at a six kilometer spatial resolution from historical incident wave data measured by the Coastal Ocean Currents Monitoring Program (COCMP) stations using High-frequency radar (HFRadar) and archived on internet databases. These models provide high temporal resolution estimates of current direction at a particular location.

Finally, note when an intermittent contamination pattern does not match any particular temporal phenomenon. These intermittent sources can be suggestive of illicit dumping or other hard-to-detect anthropogenic activity. In other cases, understanding the intermittent nature of the problem provides insight for later investigative phases to ensure source-tracking technology is deployed at times when the source is present.

Box 3.3. Example temporal data analysis using FIB

Researchers compiled FIB data from 60 beaches in Orange and Los Angeles Counties, along with information on the time of day the FIB sample was collected. Using the time of day data, they were able to use tidal prediction algorithms to determine whether the tide was rising or falling, and whether it was spring or neap tide during sample collection. The researchers binned the data according to the tidal condition and explored whether FIB differed significantly under various tide conditions using a Chi-square test or Fisher's exact test. They found tidal patterns at 50 of the 60 beaches analyzed. This led them to conclude that sources of contamination other than terrestrial runoff (including groundwater, sand, and wrack) may be pervasive at these beaches.

For more information see: Boehm, A.B. and S.B. Weisberg. 2005. Tidal forcing of enterococci at marine recreational beaches at fortnightly and semidiurnal frequencies. Environmental Science and Technology 39:5575-5593.

CHAPTER 4: (STEP 3) TRADITIONAL METHODS FOR IDENTIFYING LEAKS IN SANITARY SEWER AND ON-SITE WASTEWATER TREATMENT SYSTEMS

Verifying integrity of sewage conveyances is a top priority for investigating fecal indicator bacteria (FIB) sources to a beach because:

1) Human sewage carries a high bacterial and human pathogen load, causing it to have a high public health risk relative to non-human fecal sources;

2) Coastal infrastructure is typically more aged (since coastal areas are generally the soonest to be developed) and more subject to corrosion from saltwater contact;

3) Sanitary systems have been found to be responsible for fecal pollution at many beaches; and

4) Methods for testing sanitary system integrity are proven and typically less costly than the genetic source identification technologies used in later tiers of this manual.

California has three principal types of sanitary treatment and conveyance systems. In most developed areas, California has separate, dedicated stormwater and sewage conveyance systems. However, many communities with separate sewer systems also have on-site water treatment systems (OWTSs), such as septic systems. OWTSs are more prominent in rural areas. Finally, combined sewer overflow systems convey both sewage and stormwater together through a common pipe to a regional water treatment facility. Combined sewer overflow systems are least common in California and limited to San Francisco and parts of Sacramento. The approaches used to test for leakage differ among these systems and are described separately below.

4.1 Separate Sewage Conveyance Systems

Several techniques test the integrity of sanitary sewer systems, most prominently dye testing, smoke testing, and closed circuit television (CCTV) inspections. Dye testing involves placing dye into a portion of the system and then looking for down-system leakage of that dye into surrounding surface or groundwater. This is the most versatile method, but often not the one routinely used by many sewer system operators.[2]

Smoke testing is more complex to conduct, but has the advantage of testing many potential sources at once. Whereas dye is placed into individual source locations and then flows down the system, smoke is forced into the system and can identify up-system defects, making it particularly valuable when there are multiple lateral lines converging in an area or suspected illicit connections.

Sewer system operators most commonly use CCTV for preventive maintenance. It involves putting a camera into the conveyance system and provides a direct visual line of evidence. CCTV has the advantage of potentially identifying specific defect(s) or cross-connections when

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[2] For more information on common practices used by sewer operators in California see
http://bacwa.org/Portals/0/Committees/CollectionSystems/Library/SSO%20BMP%20Manual%201%201.pdf.

combined with dye testing. However, numerous small defects may not be visually apparent and, for source tracking purposes, CCTV is best used as a confirmatory method when dye testing or smoke testing has already identified the general origin of the leakage.

Dye Testing

Dye testing involves introducing fluorescent dye into a sanitary system to verify its integrity or identify defects. It is also used to test the function of OWTSs and detect connectivity between these systems and surface waters.

When testing sanitary collection systems, dye is added upstream of the suspected defect through toilets, sinks or directly into the pipe and then detected either visually or by fluorometry. Visual detection is standard practice and is often adequate in the case of large leaks or cross-connections to storm sewers, but dye from small leaks or dye diluted by ground or surface waters is often undetectable by sight. For this reason, use of rhodamine- or fluorescein-based dyes that are detectable in very small concentrations using a fluorometer is recommended.[3]

In tidally influenced areas, dye testing should be conducted during spring tide conditions, when tidal height fluctuates the most. During these events, the increased water flux between the land and ocean creates the best opportunity to detect dye that has escaped from the system. Baseline water samples to calibrate fluorometric measurements should be collected before dye is introduced into sanitary systems through toilets or directly into the line in question during low tide. Visual observations and water sampling for fluorometry should be initiated immediately following introduction of the dye and continue at set intervals, or continuously using instrumentation, over at least two full tidal cycles to allow time for dye to travel through the soil into the areas where measurements are taken.

Box 4.1. Example of dye testing to check integrity of a sanitary collection system

Doheny State Beach Park in Dana Point, CA had a history of poor bacterial water quality. Initial hypotheses implicated the large resident shorebird population. However, an epidemiology study linked illness in swimmers with water contact and human-associated fecal markers and viruses had been detected in beach water. This evidence suggested the sanitary sewer infrastructure near the beach might be leaking. To test the integrity of the sanitary system, rhodamine dye was introduced through toilets in restrooms surrounding the beach and the ocean and adjacent creeks were monitored for rhodamine every two hours over the next 30 hours. While the dye was never visually detected, fluorometer readings showed that the dye flowed to the ocean within hours and was widespread across the beach, indicating that there were defects in the sanitary collection system. Subsequent CCTV inspection of the gravity collection lines within the park revealed multiple defects and corrective action was initiated to restore the integrity of the system. This beach is an example of an obvious FIB source (shorebirds) masking an infrastructure problem.

For additional information see Layton et al. In preparation

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[3] Information on how to dye test sanitary collection systems can be found in: Existing Sewer Evaluation and Rehabilitation: WEF Manual of Practice No. FD-6 ASCE/EWRI Manuals and Reports on Engineering Practice No. 62, Third Edition, WEF Press: Alexandria, Virginia 2009.

Dye testing is also useful in detecting cross-connections and/or exfiltration of sanitary sewers to the storm sewer system. In this case, dye is introduced into the sanitary sewer upstream of the suspected cross-connection or point of exfiltration, as indicated by FIB data. Dye entering the storm sewer may be detected visually or through the use of a robotic camera system strategically placed in the storm sewer. In cases where sanitary sewage exfiltration into a storm sewer is suspected, exercise caution before concluding that no infiltration occurs, as dye diluted by groundwater may not be visually detectable. If this is the case, you may want to verify the result using fluorometry or a human-associated fecal marker (see Chapter 5).

Box 4.2. Example of dye testing to check sanitary sewers exfiltrating into nearby storm sewers

In Santa Barbara, CA, urban storm drains flowing into creeks during dry weather were found to carry high FIB concentrations. Further testing of storm drains in the vicinity of aged sanitary sewers showed that DNA-based markers of human waste were present. Investigators hypothesized that the aged sanitary sewers were exfiltrating into soils, and that exfiltration was migrating into nearby storm drains, i.e., storm drains underneath or parallel to leaking sewers. A follow-up study involved releasing rhodamine dye into sanitary sewer manholes at several locations that spatially bracketed the affected storm drains. An optical, real time monitoring probe with an automatic data logger (YSI Instruments) was deployed into a storm drain manhole at a location both contaminated with FIB and potentially integrative based on its location and directions of drainage and sewage flows. They deployed the probe over several days to weeks, while downloading data and inspecting it periodically. Within a one- to two-week period, they detected exfiltration via a small rhodamine dye peak within the logged probe data (Sercu et al., ES&T, 2011). This confirmed the bracketed area was problematic for sewage exfiltration, and it was prioritized for sewer slip-lining and replacement by the City. The City incorporated this dye testing method into an urban drain microbial source tracking guide published at http://www.santabarbaraca.gov/civicax/filebank/blobdload.aspx?BlobID=16722.

For more information see: Sercu, B., Van De Werfhorst, L. C., Murray, J. L. S., and Holden, P. A. 2011. Environmental Science and Technology 45:7151-7157.

You may also use dye to determine which portions of a beach are affected by flow from particular sources. In this application, introduce dye upstream of the drainage being studied. Visual observation of dye dispersal gives a good general idea of which beach areas are impacted. For a more sophisticated analysis, use fluorescence measurements of the initial dye concentration as well as water samples taken along the beach over time. Then, use this dilution estimate to calculate the likelihood that FIB from the drainage are responsible for the concentrations observed at particular locations along the beach. Dye can also be used to infer information about water movement at the impacted beach to determine residence time and thus provide insight into FIB fluxes needed to maintain specific FIB concentrations at the beach.

Smoke Testing

Smoke testing involves isolating a small section of the sewer system and then blowing smoke through that section from a manhole (Figure 4.1). If defects exist in either the main sewer pipes or the private sewer laterals that serve individual homes and businesses (Figure 4.2), or if there are unknown or illegal connections between the sewer and storm drain, smoke will rise to the surface and reveal these problems.

Smoke testing is more cumbersome, and therefore generally less desirable, than dye testing but can be valuable for evaluating multiple lateral lines into a conveyance system. Whereas dye testing would require dye placement into each home, the positive pressure associated with smoke testing forces the tracer upstream in the system and allows simultaneous evaluation of connections from multiple houses in a region. Smoke testing can also be valuable to check for cross-connections by blowing smoke into the drainage system. If there is a direct cross-connection from a particular residence or building, smoke will be seen rising from the drain vents of that structure (See example, Box 4.3).
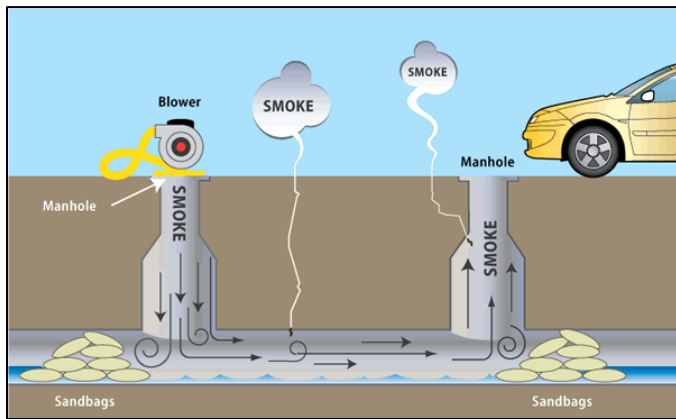
Figure 4.1. Smoke testing in a sewer main.

Figure 4.2. Smoke rising from a faulty lateral line.

Box 4.3. Example of smoke testing to identify and confirm a suspected cross-connection

High *Enterococcus* (ENT) counts resulted in repeated advisories at a recreational site on the Chesapeake Bay. Intensive sampling identified a "hot spot" in the water adjacent to a storm drain at one end of the beach. Most samples collected from both the hot spot and storm drain were positive for a human-associated fecal marker (BacHum). Long-term storm drain sampling at spring low tides, the only time the outfall was available for sampling, revealed high concentrations of *Enterococcus* (450 CFU/100 mL to >3,000 CFU/100 mL). The advisories were most frequent if sampling occurred on the outgoing tide; and investigators suspected a large home subdivided into rental apartments in line with the storm drain as the source. Denial of access by the property owner precluded a dye test, so a smoke generator and fan were used to blow smoke into the storm drain during a low spring tide. The emission of smoke from plumbing fixtures in the building demonstrated a direct connection. Repairing the sewer line from the house and connecting it to the sanitary sewer quickly reduced the ENT counts below detection limits in subsequent samplings. No beach advisories have occurred at that location since the repair was made.

Personal Communication: Dr. Charles Hagedorn, Virginia Tech, Department of Crop & Soil Environmental Sciences

Closed Circuit Television

There are two basic types of Closed Circuit Television (CCTV) systems. Both are used to perform routine and investigatory inspections on the gravity portion of sanitary and storm water collection systems, and may also help locate defects in sanitary pipes when FIB spatial patterns suggest a problem in a specific area. The first type consists of a high-pressure water jet and camera on a flexible cable. The second type uses a self-propelled robotized camera system designed to operate remotely in confined space.

The cabled system cleans debris and biofilm off the inside of pipes with a water jet so the camera can take video images of the pipes' inner surface lumen. This unit detects large defects such as offsets in pipe ends caused by root intrusions or shifting substrate. It also detects smaller cracks that, when numerous, may discharge a substantial amount of sewage into groundwater. The larger robotized system can go into confined space to inspect large pipes, but also applies to applications such as visually detecting cross-connections to storm sewers when dye is introduced into the sanitary sewer system (Box 4.4).

When a sewer pipe is inspected with CCTV, the technician should be recording observations in detail. This data can be used to prioritize needed improvements. Storm drain pipes can also be inspected using CCTV to search for evidence of cross-connections such as sewage debris or to detect dye introduced into the sanitary sewer.

While useful, CCTV systems often miss small defects. Thus, they are generally preferred as a confirmatory tool to identify specific leaks after dye or smoke testing finds a leak in the area. Alternatively, CCTV may be used as an initial screening tool, particularly for utilities that have the equipment readily available, but findings should be confirmed with dye testing when FIB concentrations suggest a concern in the area.[4]

Alternative methods

In addition to the traditional methods mentioned above for identifying leaks in sewer systems and OWTSs, new tools are also emerging. One example is the use of canine scent tracking. Canines excel at scent detection and scent discrimination, and are able to focus on their trained target and ignore other sources. Canines have been trained and used successfully to detect a wide variety of scent sources (e.g. pests, imported species, some types of cancer, etc.). In recent years, canines have also been trained to detect municipal wastewater, and initial field evaluation of the method appears promising (see Box 4.5.).

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[4] Find specific information on the use and proper operation of CCTV systems at:
http://bacwa.org/Portals/0/Committees/CollectionSystems/Library/SSO%20BMP%20Manual%201%201.pdf.

> **Box 4.5. Using canine scent tracking to screen for human waste pollution**
>
> An evaluation of canine scent tracking as a field approach for surveying human fecal contamination was conducted in Santa Barbara, CA. Two dogs had been trained to respond to the scent of municipal wastewater. In this study, canine response to water inside storm drains was later compared to results from fecal indicator bacteria (FIB), and chemical and DNA-based human fecal marker assays. The dogs responded positively (70% and 100%) at field sites where water samples were later confirmed in the lab as contaminated with human feces. Further, when both dogs indicated a negative response, tests for human fecal markers were negative. An advantage of canine scent tracking is that it provides instant, on the ground results. For example, in this study the dogs were used for a storm drain diversion structure to indicate the upstream location of human fecal contamination. The dogs surveyed all upstream branches of the storm drain system and quickly located a likely origin of contamination related to a surcharging sanitary sewage lift station. Visual evidence of the surcharge entering the adjacent storm drain was gathered using televising and dye studies. Human waste was then confirmed for sampled storm drain water using the human-waste assays. Canine scent tracking is a promising tool that may be useful for screening and prioritizing sampling sites before DNA-based and other similarly expensive assays are used to confirm human fecal contamination.
>
> *For more information see: Van De Werfhorst, L. C., Murray, J. L. S., Reynolds, S., Reynolds, K., and Holden, P. A. Canine scent detection and microbial source tracking of human waste contamination in storm drains, In press, Water Environment Research.*

4.2 On-Site Water Treatment Systems

Once the location of OWTSs are identified and cross-referenced to FIB data in the region to identify the most suspect OWTS sources, dye testing is the preferred option for investigation. Standard practice looks for pooling of dyed water on the surface or entering waterways, but like sanitary sewer dye testing, fluorometry boosts sensitivity.[5] Dye pulse size and delivery mechanisms, leach field size, soil type, and soil permeability all affect dye testing of OWTSs. For best results, an experienced hydrology team should conduct these tests.

Self-contained OWTS units are generally located entirely on private land and the local landowner may not grant access to their system for introducing dye. Some municipalities have ordinances that compel landowners to allow local officials to inspect OWTSs after proper notice. In areas where such ordinances do not exist, local public health officials and Water Boards are empowered to force inspection and may disallow use of the system, but only after a known or suspected public health threat or a connection to contaminated surface waters has been identified.[6]

If a landowner refuses access, then indirect offsite methods would be necessary to investigate the source. In locations with sparse OWTS density and unlikely potential for other human sources in the area, this typically means using either a human genetic marker and/or chemical testing as outlined in Chapter 5. In areas with a high OWTS density, or other potential alternative human fecal sources, the more complex community based methods outlined in Chapter 7 may be useful.

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[5] Find information on general procedures to dye test OWTS at: http://www.stahi.org/septic-dye-test-protocol.html. For detailed technical information on this topic see: Lalor, M. and Pitt, R. 1999. Use of Tracers to Identify Sources of Contamination in Dry Weather Flow. *Watershed Protection Techniques* 3(1).

[6] For additional information on the State Water Resources Control Board's OWTS policy see: http://www.waterboards.ca.gov/water_issues/programs/owts/board_adopted_policy.shtml

Box 4.4. Identifying leaking sewer laterals with a robotic camera and fluorescent dye

During a routine survey of Mission Creek in Santa Barbara, degraded toilet paper and brown sludge were observed in water flowing from a storm drain. In addition, FIB counts from outfall samples were consistently high and "sewer corn" kernels were frequently observed at the outfall. Investigators hypothesized that fecal pollution was entering the storm drain directly and tested it using two tools: a camera and a fluorescent dye. They first inspected the storm drain interior using a CCTV camera mounted on a robotic vehicle (*below left*). Images showed the old drain had a brick and mortar roof, plus flowing water (*below right*). Suspicions of sewage input were confirmed when the camera found two locations with water dripping into the storm drain.

Although the evidence pointed to a sewer leak, it was difficult to visualize how solids such as toilet paper and baby wipes could be forced through the small crack. The second step tested for connections by adding fluorescent green dye to the toilets of nearby businesses. After a few flushes, a bright green stream flowed out of the storm drain and into a creek (*above left*). With the camera in the storm drain trained on the dye, a large void appeared that allowed solids to enter the drain. A similar procedure identified a second leak, and private property owners replaced the leaking sewer laterals with new pipe (*above right*) within 24 hours of confirming the source. Following the repairs, no water flowed from the drain outfall, demonstrating proper identification and remediation of the fecal pollution source.

CHAPTER 5: (STEP 4) INDICATORS OF HUMAN FECAL POLLUTION

Source identification efforts typically place the highest priority on evaluating whether human fecal material contributes significantly to the FIB load, as human fecal material contains a greater number of human pathogens than other fecal sources and thus represents the greatest potential health threat. The traditional technologies in Chapter 4 less effectively identify small leaks from sanitary sewers, undocumented pipes, or leaking septic systems, as well as direct deposits potentially associated with a homeless encampment, leading to a need for alternative technology.

This phase of investigation involves measuring human-specific source markers. These markers detect regions of genetic material in fecal bacteria associated with human hosts, using quantitative polymerase chain reaction (qPCR) techniques. These methods demonstrate both sensitivity and specificity to human fecal material. While bacterial markers usually suffice to verify the presence of human material, in some circumstances results may require supplementary confirmation with either direct human pathogen measurements (e.g., viruses) or chemical markers. The remainder of this chapter describes the appropriate tools in each category and explains when use of more than one tool might be warranted.

5.1 Bacterial Markers

Human-associated bacterial source markers are emphasized at this stage not only based on their superior performance characteristics, but also due to their relatively low cost and ease of use. The qPCR technology has been widely used for medical applications in public health laboratories for many years. In addition, the US EPA recently released a qPCR method as a tool for rapid measurement of FIB in beach water samples. While buying the equipment and setting up a lab to perform qPCR requires an initial capital investment (approximately $100K), the subsequent cost for supplies totals roughly $50 per sample and a competent technician can process more than 40 samples in one day (following DNA extraction). (A commercial laboratory may charge tens to hundreds of dollars per sample; prices will vary depending on the company, location, and number of samples.) Moreover, the EPA is developing a standard method for human-associated qPCR markers, based largely on the standard operating procedures (SOPs) developed during the SIPP method evaluation study (Appendices A4 and A5). These factors will likely increase access to the technology.

Researchers have developed more than a dozen human markers over the last decade, but one of these, HF183 Taqman®, performed best overall in method evaluation studies.[7] A slightly less sensitive marker, HumM2, also performed well. Both of these markers target *Bacteroides* bacteria in human fecal material. This group of bacteria consistently exhibits host-associated gene sequences. In evaluating human-associated markers, studies have evaluated method sensitivity (i.e., does the method detect human material when it is present in the sample?) and specificity (i.e., does it also detect other fecal sources?).

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[7] Layton, B.A., Cao, Y., Ebentier, D.L., et al. 2013. Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. *Water Research* 47 (18), 6897–6908.

The HF183 Taqman marker (Appendix A4) is the best starting point for detecting human fecal material because it provides the best combination of sensitivity and specificity. However, although it performs highest among other markers on sensitivity, it has been shown to occasionally detect ("cross-react" with) chicken or dog feces. If those sources pose a concern in the watershed, or if managers simply desire to add certainty about the results, HF183 can be paired with HumM2.

5.2 Human Viral Markers

While human-associated bacterial markers typically suffice for this phase, the source identification effort may warrant use of an even more specific marker, for example when cross-reactivity of human-associated bacterial markers occurs. The high cost of mitigation might also justify additional verification testing to confirm the presence of human fecal material, particularly municipal sewage.

Human virus measurement represents an independent approach to verify human bacterial marker results. Several methods detect viruses excreted in feces and/or urine with high specificity to human waste and almost no cross-reactivity with other sources. The downside of these methods is relative insensitivity, despite continuing efforts by researchers to improve them. This problem stems from very low-density occurrence of viruses in the environment and difficulty concentrating them efficiently with current technology. Detection may be enhanced by concentrating large volumes of water, up to 1000 times more water than the 100 mL sample typically used for bacterial source markers, but this adds logistical challenges and expense as water must be processed on-site or transported to the laboratory in large volumes. Thus, the additional cost of concentration, which adds hundreds of dollars per sample, and logistical challenges of measuring viruses must be weighed against the cost of remediation and the weight of evidence needed.

Among the virus methods, markers for DNA viruses, such as human adenovirus and human polyomavirus, are among the more sensitive and robust. These viruses are fairly widespread among humans, and a sizable portion of the population sheds polyomaviruses passively. In addition, the DNA genomes of these viruses are less labile than those of common human enteric viruses with RNA genomes, which may make them more resistant to environmental degradation and therefore easier to detect. However, due to the specialized steps required and ongoing research to improve concentration and recovery of viruses from water, the authors recommend that only an experienced research laboratory perform these methods.

5.3 Chemical Source Tracking

A third independent approach to evaluate environmental water for presence of human fecal material measures chemicals relatively specific to wastewater and septage. Such chemicals include optical brighteners used in laundry detergents; caffeine; fecal sterols, which are metabolic byproducts of human digestion processes; and a metabolite of nicotine (cotinine) excreted by tobacco users. However, these methods are rarely used because their analytes are quickly diluted to levels below detection limits once the waste stream enters the ambient environment. Moreover, the cost to measure the organic chemicals, such as sterols, surpasses that of traditional cultivable bacteria indicators. However, for some laboratories (e.g., sanitation districts) with the necessary instruments for detailed chemical analysis, chemical methods cost

less than qPCR-based assessment of human waste DNA markers. Thus, some chemical markers might be useful for independently confirming human waste inputs in a cost-effective manner.

One chemical source tracking method previously used in California is the Enzyme-Linked Immunosorbent Assay (ELISA) test to detect caffeine and cotinine. The ELISA technique involves a biochemical assay to detect the presence of an antibody or antigen in a sample. Kits to measure caffeine and cotinine are commercially available, reasonably priced, and easy to use. An additional aliquot of the sample can be filtered in the field during sample collection for FIB and DNA-based analysis. The filtrate can then be stored frozen until investigators have decided whether or not to analyze for chemical markers.

Box 5.1. Using caffeine and cotinine markers to confirm human waste in storm drains flowing to coastal creeks during dry weather

The lower Laguna watershed in Santa Barbara, CA, had high concentrations of FIB in creek locations flowing into a coastal lagoon and then to the beach. Upstream storm drains tested during dry weather carried sewage contamination as indicated by DNA-based human markers. At locations with high bacterial indicator concentrations, caffeine and cotinine concentrations correlated with one another as well as with DNA-based markers of human waste. These positive results for chemical evidence of human waste provided further confirmation that human inputs, and not cross-reaction of PCR primers with other fecal sources, were causing high DNA-based human markers in the drains. The locations of highest contamination were further investigated to locate possible origins. While exact spatial sources were not determined, the City prioritized this area for sewer rehabilitation and initiated the project.

For additional information see Holden, Patricia et al. In preparation

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Another chemical detection tool, optical brighteners (OBs), may be useful in rare circumstances to identify water flowing from straight pipes or septic systems. The advantage of OBs is easy measurement in the field with a handheld fluorometer, providing near real-time and inexpensive signal tracking, which cannot be said of any other method above. This allows an investigator to walk a stream, test the water at periodic intervals, and sample adaptively based on presence of the OB signal to better geographically pinpoint the source. However, OB detection is not as sensitive as most microbial assessments, and real-time source tracking efforts will succeed only if the OB concentration produces a measurable signal. Additionally, OBs may be absent from households using certain environmentally friendly detergents that do not contain these additives. Because of these limitations, OB analysis is mainly useful as an additional piece of information to achieve a greater weight of evidence and should not be relied upon as the sole detection method for human fecal pollution.

Nitrate is another chemical marker sometimes useful in diagnosing flow from septic systems into surface water. In a properly working septic system, almost all nitrogen entering the soil is quickly converted to nitrate by soil microbes. Despite the fact that nitrate is always found in septic discharges and relatively cheap to measure, the presence of nitrate in surface water does not guarantee that it came from a septic system, as it is also found in high concentrations in

runoff containing fertilizers applied to crops and lawns. Further, atmospheric deposition from combustion of fossil fuels can also introduce nitrate to surface waters. Therefore, one should think twice before considering nitrate as a source tracking indicator in areas with known aerial deposition, such as the Los Angeles metropolitan area. As with OBs, the presence of nitrates must be considered only as another piece of information to add to the weight of evidence, rather than an irrefutable confirmation of connectivity between septic tanks and surface waters.

CHAPTER 6: (STEP 5) NON-HUMAN SOURCE-ASSOCIATED MARKERS

Employing non-human source-associated markers is the next step when source identification tiers 1–4 (FIB data, infrastructure investigations, and human-associated markers) have suggested that human fecal contamination is not the dominant source. Only a limited number of the most common species have source-associated markers available, but many feature both sensitivity and specificity for their targets. This chapter provides guidance for selecting and applying these tools.

6.1 Selecting Appropriate Markers

Reliable source-associated fecal markers are currently available for cattle, dogs, pigs, horses, and gulls. Other, more general markers target ruminants and other waterfowl. Selecting which non-human source-associated marker to use depends on the hypothesized sources identified during the information-gathering phase of the study (Chapter 2). Practitioners can choose one or multiple markers as appropriate to answer the question(s) at hand. To save cost, stored DNA extracts used to conduct analysis for human-associated markers may be re-analyzed with additional markers. Additional samples may need to be collected to ensure adequate spatial and temporal coverage.

Given a limited budget, you must balance the need to collect an adequate number of samples with the expense of gaining information about an additional source. Take care to run multiple markers only when host sources are numerous enough in the watershed to warrant the added effort and expense. For example, combining non-human markers would be helpful at an off-leash dog beach that also has a resident gull population (Box 6.1). In this case, managers might hypothesize that both sources are contributing FIB to the water and seek to better isolate the most significant source.

Box 6.1 Use of multiple nonhuman markers to identify significant FIB sources

Sampling at Arroyo Burro Beach in Santa Barbara, CA revealed an intermittently breaching lagoon as the dominant source of dry weather FIB to the surf zone. Human sources in the lagoon and downstream creeks feeding the lagoon were ruled out using the HF183 and HumM2 markers. The DogBact and Gull2 markers implicated dogs and gulls as the likely nonhuman sources at this beach. While gull markers were detected at low levels in the surf zone and lagoon, dog markers correlated highly with FIB in the surf zone. Dog markers were also present in the lagoon and at a downstream creek location. Load calculations revealed that dog markers measured in the surf zone and lagoon came primarily from the upstream creek. Follow-up actions taken by the City of Santa Barbara helped to limit the input of dog feces from homeowners living along this creek.

For additional information, see Ervin et al. In preparation.

6.2 Choosing Appropriate Non-Human Source-Associated Fecal Markers

All markers described in this chapter use PCR. The majority of markers use quantitative PCR (qPCR), meaning they provide information about the relative amount of target material in a particular water sample. Others, termed "end-point" or conventional PCR, provide only qualitative (presence or absence) data. When quantitative and end-point PCR assays both exist for a particular source, the quantitative assay is always preferred. Aside from providing quantitative information about the target in a water sample, the design of qPCR assays adds additional sensitivity and specificity that is not available in non-quantitative PCR methods.

This section describes recommended markers selected from among the suite of non-human source-associated markers currently published for use in MST. It relies heavily on the results of the SIPP MST Method Evaluation Study, which tested method performance with fecal material collected throughout California, although other validation data was also taken into consideration.[8]

Dog-Associated Fecal Markers

At present, several dog-associated qPCR and end-point PCR assays have been published. Two of these have been evaluated for use in California, DogBact and BacCan-UCD, and perform equally well when dogs are a potential fecal source. Though highly sensitive and specific, both methods occasionally cross-react with other species. Find the SOP for these two recommended methods in Appendix A6 and A7.

Cow/Ruminant Markers

Multiple cow- and ruminant-associated DNA marker assays have been developed in the last decade and several evaluation studies have been conducted to validate their performance. Three qPCR assays have consistently performed well in locales around the world: BacR, CowM2, and Rum2Bac. Among these, CowM2 is the recommended cattle marker because it will become an EPA-approved method in the near future. When you suspect a non-bovine ruminant fecal source, such as deer or goats, use Rum2Bac instead of CowM2. When both cattle and other ruminants live in the watershed, apply both CowM2 and Rum2Bac. Rum2Bac occasionally demonstrated false positive results with septage, so in watersheds with both septage and ruminant sources, conclusively rule out septage before employing Rum2Bac. Find the SOPs for CowM2 and Rum2Bac in Appendices A8 and A9, respectively.

Pig Markers

Relatively few pig-specific markers have been developed to date. Two pig markers, one based on conventional PCR (PF163) and one quantitative (Pig2Bac), have been tested for use in California. Due to its demonstrated high sensitivity, Pig2Bac is the recommended method for detection of pig feces. Note that Pig2Bac sometimes cross-reacts with human/septage and dog

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[8] Detailed information about the SIPP study can be found at
http://www.sciencedirect.com/science/article/pii/S0043135413005496.

feces, so it works best when those sources have been ruled out. Locate the SOP for this method in Appendix A10.

Horse Markers

Horse-associated markers are relatively uncommon. To date, only two have been published and only one conventional PCR-based horse marker (HoF597) has been validated in California. Its use is recommended when horses are present in the watershed and all other potential sources have been ruled out. It performed well in the SIPP study, but does not show as much sensitivity as most other host-associated assays. The other recently developed quantitative horse assay has not yet been thoroughly evaluated. As such, the recommended conventional horse marker SOP is given in Appendix A11.

Gull Markers

The MST toolbox gained host-associated markers targeting gulls and other water birds fairly recently. Selecting from a number of sea bird-associated assays published to date, the SIPP study evaluated four for use in California. Of these four, the Gull2 Taqman and LeeSeaGull markers are best for routine use because they consistently displayed sensitivity and specificity across laboratories. All available bird markers will detect pigeon and sometimes goose feces as well as gull. Further, the host distribution of the bird marker target bacterium (*Catellicoccus marimammaliam*) among all the various species of shorebirds is unknown. Thus the Gull2 and LeeSeaGull markers should be considered general "bird" assays and not necessarily specific to gulls. Several new bird-associated MST assays developed since the SIPP study was conducted may prove superior to Gull2 and LeeSeaGull, but require a thorough evaluation. Therefore, the two evaluated and recommended Gull2 Taqman and LeeSeaGull SOPs are presented in Appendices A12 and A13.

CHAPTER 7: (STEP 6) MICROBIAL COMMUNITY ANALYSIS

Microbial community analysis (MCA) methods can either complement the techniques described in the previous chapters, or fill in gaps where the prior methods do not achieve the intended MST goals. However, they remain in a somewhat experimental state as only limited studies illustrate their utility in field settings. Unlike the single marker methods (in Chapters 5 and 6) that measure one microorganism or group of microorganisms at a time, MCA uses a set of tools to simultaneously provide information about the entire microbial community present in a sample. MCA can operate in a library-dependent fashion, where patterns in environmental samples are compared to a "library" of local reference samples taken from suspected sources, or a library-independent fashion, in which profiles among environmental samples are compared to discern spatial and temporal gradients.

MCA offers added benefits in MST scenarios where:

1) Single-source markers have not yet been developed for a suspected fecal source (e.g. chicken, deer, or goose). In the absence of a host-specific marker, MCA may match the pattern of a hypothesized source to the pattern in DNA of sampled waters. Because MCA methods first characterize the microbial communities in the suspected sources and then compare them with unknown sample communities, their source identification capacity is not restricted to the single marker methods already developed, or by the need to perform multiple single marker assays for multiple sources. The suspected source can be any source for which a reference sample can be obtained.

2) A host-associated marker spans multiple sources that need to be distinguished for management purposes. For instance, when a human marker is found in the watershed, practitioners may seek to determine whether the fecal material originated from sewage, septage, or stool (e.g., a homeless encampment). An MCA analysis may make this differentiation possible because, according to laboratory analyses by SIPP and other studies, the overall microbial communities differ among these three human sources.

3) Further evidence (other than FIB detection) is needed to determine if non-fecal sources are major contributors of FIB loading to water. Microbial communities originating from non-fecal sources such as storm drain biofilms, sand, and algal wrack could be compared to those found in the receiving waters. High similarity would provide added evidence that the microbes in the water originated from the sand or wrack, for example, rather than from fecal sources. However, neither laboratory validation nor field demonstration has been conducted for this application.

4) Further evidence (other than relative magnitudes of FIB or marker signals) is needed to identify spatial gradients of fecal pollution. Microbial community analysis integrates source types and loading information using multivariate data analysis and display approaches, allowing observation of the influence of waste inputs on receiving waters over space and time. The influence of waste inputs on receiving water community composition attenuates with distance away from the physical location of the fecal source, as demonstrated in a MST field study in Santa Barbara (see Box 7.1).

While MCA may complement single marker methods in these scenarios, its application to MST is still in its infancy, making it more appropriate as a late tier tool. Some MCA limitations include:

1) Dramatically higher cost. The processing cost per sample exceeds that of methods described earlier since extra laboratory steps remain after performing basic PCR. In addition, it carries a higher labor cost because MCA data analysis requires a high level of expertise and/or computing power.

2) Less accessibility. While many local processing laboratories now have the qPCR technology required to perform single marker methods (which matches the EPA-approved technology for rapid FIB processing), most labs do not have the microarray readers or genetic sequencing equipment required for MCA. Moreover, the advanced data analysis methods to interpret community data often require specialized software and training. In addition, fewer field studies have modeled MCA application retrospectively. As such, most MCA applications at present will require collaboration with a research laboratory, which may add cost and delays in developing contractual relationships.

3) Lower sensitivity. Low-resolution community profiles (such as those provided by fingerprinting methods) usually do not detect minor fecal sources influencing the environmental water samples. This relatively low sensitivity makes MCA methods inappropriate for management applications where high analytical sensitivity is preferred, e.g., for detecting very low levels of human waste. Although high-resolution community profiles, (such as those provided by PhyloChip or, even better, next-generation sequencing,) may achieve higher sensitivity, researchers are still actively developing suitable bioinformatics tools that can mine such high-resolution profiles.

Even with these limitations, MCA represents a very dynamic research area experiencing rapid development in many aspects: improved molecular technology to simultaneously measure the entire community with high resolution, bioinformatic tools enabling faster and more sophisticated data analysis, and more field implementation case studies. These developments will likely reduce cost and improve accessibility and sensitivity, imbuing MCA with the potential to become a universal MST tool for all sources. Nevertheless, due to data comprehensiveness and analysis complexity, MCA will still need to be conducted in collaboration with an experienced research lab in the foreseeable future. The following sections of this chapter will expand on describing the existing MCA technology and considerations for using MCA in MST.

7.1 Types of Microbial Community Analysis Methods

Three types of microbial community analysis methods have been tested for MST: community fingerprinting, such as terminal restriction fragment length polymorphism (TRFLP); microarrays such as PhyloChip; and next generation DNA sequencing, such as Illumina sequencing. While all three approaches rely on DNA amplified by PCR to assess microbial community composition, they differ in the technology used for detection and level of resolution. Even more than for single marker methods, data analysis is an integral part of MCA methods, and data analysis choices greatly affect how well MCA performs for MST. A simple flow diagram depicts the MCA MST process in Figure 7.1. The following paragraphs describe each MCA method separately.
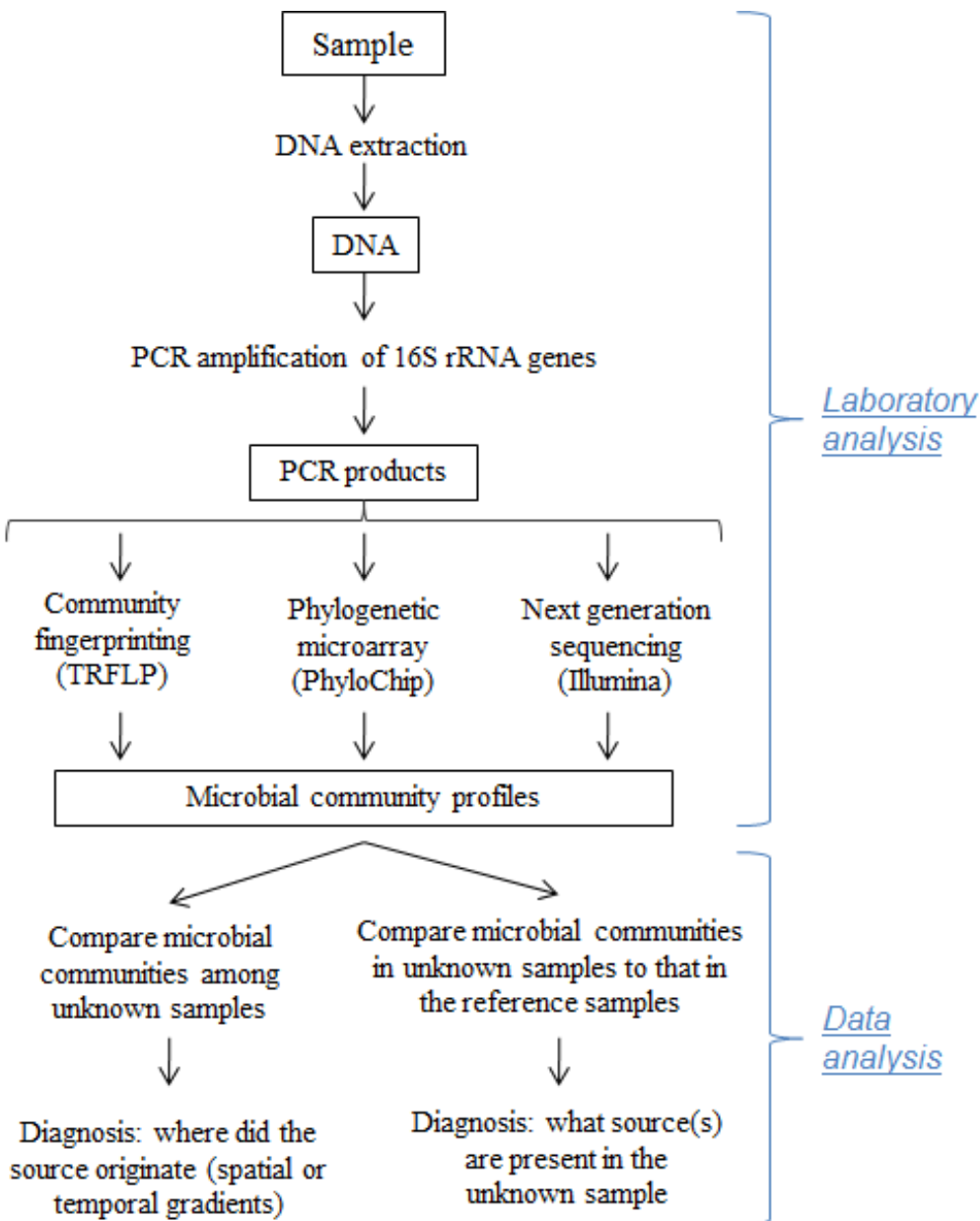

Figure 7.1. Flow diagram illustrating the process of using MCA for MST.

Box 7.1. Example of a TRFLP integrated community analysis approach

East Beach in Santa Barbara, CA, had several potential FIB sources, including a creek with a tidal lagoon and dry weather flow from a storm drain that was previously determined to contain human contamination. Using TRFLP, the bacterial communities from environmental samples were compared to reference samples from fecal sources in the watershed (sewage; human, gull, and raccoon feces). Statistical analysis clearly distinguished the microbial communities of each sources, revealing a spatial and compositional gradient (i.e., ocean to lagoon to urban creek/storm drain to sewage), and confirming the presence of human contamination in samples positive for a human-associated qPCR marker.

For additional information see: Cao. Y., Van De Werfhorst, L.C., Sercu, B., Murray, J.L. S., and Holden, P.A. 2011. Application of an integrated community analysis approach for microbial source tracking in a coastal creek. Environmental Science & Technology 45 (17) 7195-7201.

PhyloChip uses a proprietarily manufactured DNA microarray chip containing 1.1 million probes that target all known bacterial and archaeal taxa. When a matching DNA sequence binds to a probe, it fluoresces, producing a detectable signal. The pattern resulting from sample DNA bound to the probes can be used to identify bacteria and archaea (at various taxonomic levels), including some potential pathogens. This method uses multivariate statistical methods (like TRFLP), plus a customized host identifier data analysis approach, to detect reference patterns or host-specific taxa in water samples.

Box 7.2. Application of PhyloChip to discriminate between fecal pollution sources

Two field scenarios with known contamination sources were used to evaluate the application of PhyloChip for source identification in marine waters. The first was a 720,000 gallon sewage spill in Richardson Bay (Sausalito, CA). The second was at Campbell Cove (Bodega Bay, CA), a recreational beach where previous MST results indicated contamination likely came from gulls. Samples that exceeded regulatory enterococci limits (>104 MPN/100 ml single sample, >35 MPN/100 ml geometric mean) were analyzed via PhyloChip to determine if they contained previously identified microbial taxa associated with humans, birds, or grazing animals. The Richardson Bay results detected almost all of the human identifier taxa and very few bird or grazer identifier taxa. Campbell Cove findings showed very few grazer or human identifier taxa but found a significant level of bird identifier taxa.

For additional information see: Dubinsky, E.A., Esmaili, L., Hulls, J.R., Cao, Y., Griffith, J.F., and Andersen, G.L. 2012. Application of phylogenetic microarray analysis to discriminate sources of fecal pollution, Environmental Science & Technology 46 (8), 4340-4347.

Next-generation sequencing (e.g., Illumina, pyrosequencing) identifies patterns and abundances of microbial DNA sequences in reference samples, then detects these signatures within the more diverse community present in environmental water. Technological improvements in this field continue to boost the number of sequence reads produced from an individual sample. These improvements have the potential to drastically increase resolution by incorporating many millions of reads per sample.

> **Box 7.3. Use of next generation sequencing to determine fecal sources in a watershed**
>
> In this example, researchers used next generation sequencing to identify patterns in key taxa from human and animal fecal material and search for their presence in freshwater samples from the Yeongsan river basin (Jeonnam Province, South Korea). Sites included an urbanized agricultural location, a location with no major industrial influence, and a typical agricultural location. Analysis revealed the likely sources of fecal contamination to be swine and humans.
>
> *For more information see: Unno, T., Jang, J., Han, D., Kim, J.H., Sadowsky, M.J., Kim, O.-S., Chun, J. and Hur, H.-G. 2010. Use of barcoded pyrosequencing and shared OTUs to determine sources of fecal bacteria in watersheds. Environmental Science & Technology 44 (20), 7777-7782.*

7.2 Current State of Development and Method Recommendations

Application of any MCA method comes with trade-offs, as each has its own advantages and drawbacks. Of the three methods discussed in the prior section, TRFLP is the most economical and well understood, both in terms of the technology needed to perform the method and the level of expertise and computing power required to conduct data analysis. It is also the coarsest method in terms of its ability to resolve sources from complex samples, as it does not provide any additional information beyond the length and abundance of each DNA fragment. However, a recently developed iteration of the method using an additional primer set targeting Bacteroidales has enhanced the resolution of TRFLP as a source identification tool.

DNA microarray technology, such as that used for PhyloChip, is also fairly well studied. The number and redundancy of the probes provide high resolution and the ability to detect multiple sources in complex samples. However, the method costs more than TRFLP and, given a limited budget, could restrict users to a more limited reference database and reduced number of analyzed samples. Further, only two laboratories currently perform the method (one research and one commercial), which may hinder access.

Next-generation sequencing, the newest of the MCA methods, has the greatest potential to resolve multiple sources in complex samples. However, the next-generation sequencing field is still rapidly evolving. While producing millions of sequence reads per sample costs relatively little, data analysis currently creates a bottleneck. Computing power and bioinformatics expertise in working with very large and complex genomic datasets mainly exist in research laboratories. Despite these limitations, as computing capacity and the ability to automate data analysis improve, this technology will likely usurp the other methods.

All three of these methods performed nearly equally well in the SIPP method evaluation study, but the research team presently recommends TRFLP. This method is the least expensive of the three options, yet yields an acceptable level of resolution. Other techniques are likely to become more automated and cost-effective over time. Application of MCA will most likely require partnering with a research laboratory with specialized expertise. This type of partnership will provide further advice as to changes in the field and the most cost-effective technology.

CHAPTER 8: STUDY DESIGN

Designing a well-reasoned plan for where and when to sample rivals the importance of method selection in achieving an accurate study outcome. No single study design will apply to all circumstances and levels of certainty. In fact, in some cases the low cost of potential management solutions makes it more cost-effective to implement them on a trial basis before spending extra money on monitoring to add certainty about the source. Alternatively, a comprehensive design involving multiple phases and MST methods may be required if the outcome has compliance or litigation implications. While study design will differ based on the circumstances, a sound design arises from several guiding principles as described below.

In most cases, an MST study should proceed with an iterative or phased sampling approach. Evaluate hypotheses one at a time, starting with those most relevant to public health protection. After ruling out a hypothesis, conduct additional analyses or sampling to test the next most likely hypothesis. The SIPP case studies (at Cowell Beach, Arroyo Burro Beach, Topanga State Beach, and Doheny State Beach) serve as examples of this phased approach; find references to these and other field studies in the Further Reading section of this manual.

8.1 Sampling Plan Design

When developing a study plan, seek to answer five key questions:

1) How many samples should be collected?

2) Which locations should be sampled?

3) When should sampling take place?

4) Which analytical methods should be used?

5) What level of redundancy of markers is required?

The number of samples to collect represents a clear trade-off between expense and information certainty. For example, the number of samples needed to demonstrate the presence of human fecal contamination is much lower than that required to prove its absence. Only a few positive results will statistically verify a human signal, while demonstrating its absence (proving the negative) requires a much larger sample size. Addressing a question about absence relies on a binomial distribution, which identifies the number of samples necessary to confidently predict that the percentage of samples containing human fecal material is truly less than a desired level. The number of samples required will be highly specific to each beach, and the mathematics behind demonstrating absence of a source is still an area of active research.[9]

Sampling site locations largely depend on the hypotheses developed in Chapter 2. In general, the confluence of any creek, river, or drain flowing across the beach to the ocean should be sampled, along with any obvious sources or "hot spots" identified during the information-gathering stage

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[9] For further guidance on the number of samples necessary to achieve statistical certainty for a variety of scenarios, see: Cao, Y., Hagedorn, C., Shanks, O.C., Wang, D., Ervin, J.S., Griffith, J.F., Layton, B.A., McGee, C.D., Riedel, T., Weisberg, S.B. Towards establishing a human fecal contamination index in microbial source tracking. Accepted at the International Journal of Chemical and Environmental Engineering Systems.

of the study (see Chapters 2 and 3). If no "hot spots" exist, start with the most obvious physical sources, for example, the restroom on the beach or aged sanitary sewer collection lines. Sampling sites can also be chosen along a potential contamination gradient, such as several sites along a creek between a flowing drain and the ocean confluence.

Consider at least three temporal factors in deciding when to collect water samples. First, think about whether high FIB corresponds to particular flow phenomena (e.g., rain events) or occurs year-round. Second, examine the tidal cycle. Contaminated groundwater, for example, more likely flows to the beach at low tide, and a spring low tide may accentuate its influence. Third, consider seasonality. For instance, septic tanks are less likely to flow laterally to the creek or beach in the dry months when the water table is low. Conversely, these exact conditions make leaks from sanitary collection systems more detectable. In all cases, capture the conditions relevant to the hypotheses under investigation, and tailor the timing of sample collection to maximize the odds of detecting any potential human contamination sources.

If a model of FIB concentrations at the beach is to be developed to gain insight into the potential for different sources to contribute, temporally intensive sampling will be required and potential sources identified during the study will also need to be sampled.

8.2 Cost Considerations

The overall cost of a study will be dictated by the number and type of samples needed to adequately characterize a particular site. However, per sample costs for MST methods become more expensive as the technology employed becomes more complex. For example, while the per sample cost for a dye study is relatively low (tens of dollars), qPCR analysis for MST markers may cost $100 - $300/sample, depending on the number of samples and whether the analysis is conducted in-house or run by a consultant. Further, per sample costs for the more complex MCA methods range from hundreds to thousands of dollars per sample, depending on the method chosen and the complexity of the data analysis required.

Despite the relatively high cost of performing molecular analyses for MST, there are opportunities for cost savings. For example, when collecting samples for FIB analysis, practitioners often create frozen filters that are archived for later analysis. The added incremental cost is small compared to collecting new water samples and the FIB data may often be useful in guiding which of these samples to analyze using molecular techniques. Similarly, purified DNA may be stored and analyzed for additional source-associated markers without incurring additional sample collection and DNA purification costs, thereby drastically lowering the per marker cost of analysis.

8.3 Human Marker Considerations

The number of human markers to apply depends on the level of certainty required to confirm or refute the presence of a human source. In most cases, HF183 Taqman suffices, and sampling at more places and times has greater value than reconfirming certainty about human fecal presence using a different marker within a single place and time. However, if one goal of the MST effort is to provide information for development of a site-specific water quality objective, then multiple markers may add an additional layer of certainty regarding the absence of a meaningful human fecal source. Regardless of the number of markers used, always archive DNA extracted from the

original filter; likewise, create and freeze additional filters for potential later analysis. Replicate sample filters cost relatively little to create and can be archived at -80ºC for an extended period. Thus, investigators can perform additional analyses later in an adaptive fashion, for instance to obtain more information about conditions at a particular place and time, or if cross-reactivity concerns arise at a later stage in the source identification process.

8.4 Nonhuman Marker Considerations

Due to confounding factors such as the dilution and degradation rates of fecal bacteria targeted by these methods, detection frequency takes precedence over the magnitude of signal detection. As previously noted for human markers, relatively few positive samples can demonstrate the presence of a particular source, while many negative results are required to demonstrate its absence. Therefore, practitioners should opt to run only a few different markers that target the suspected sources on many samples, rather than running many different markers on just a few samples.

When several different non-human sources exist in the watershed, prioritize their investigation according to the health risk associated with each host. For example, cattle feces are regarded as the most pathogenic to humans, second only to human waste. Thus, if cows are present in the watershed, deploy cow qPCR markers ahead of any other non-human markers. Bird waste (gull, chicken) is considered less pathogenic than the feces of mammals, although the volume of waste produced by a large flock may be sufficient to pose a health risk.

While several good MST markers exist for the major non-human sources, these methods have some limitations. For example, not all fecal hosts (e.g. chicken, marine mammals) have corresponding validated markers or will have markers developed in the near future. In addition, practitioners may not be able to discern the dominant source. For example, 100% of samples may contain both gull and dog markers. The relative magnitudes of the qPCR signal cannot be used to determine which source is dominant, because qPCR signal magnitudes are unreliable (due to dilution, degradation, and differential concentrations of markers between species). Microbial Community Analysis (MCA), as described in Chapter 7, can potentially solve each of these problems: it may be able to differentiate among all possible fecal hosts and provide information about the dominant fecal contamination sources in the watershed. However, this technique has not been widely field-tested.

8.5 MCA Considerations

As with single marker methods, determining where and when to collect samples rivals the importance of method selection in reaching a proper source attribution conclusion. Many design considerations for MCA mirror those described for single-source markers, such as having appropriate temporal and spatial allocation relative to the potential sources. However, depending on the implementation approach, additional study design considerations will relate to creating the reference database (source samples).

When implemented in a library-dependent fashion, practitioners need to consider both the number of sources and number of replicates for each source type to include in the reference database. It will be tempting to collect a small number of reference sources to limit the logistical challenges and cost; nevertheless, make a concerted effort to gather samples from all potential

candidates. Too few reference fecal sources may fail to capture the range present in the watershed and result in false conclusions.

Also consider how many different samples of a particular source type to gather. For example, substantial differences in genetic signatures may exist in cattle from different farms based on the different breeds of cow, type of feed, and antibiotic regimen. At one level, these differences offer a tremendous advantage in pinpointing the source to a particular farm operation. However, it can also pose a difficulty if intraspecies variation confounds interspecies source comparisons. In general, collecting samples from several distinct populations of each host animal in the watershed and a number of individuals within each population will allow adequate quantification of intraspecies variation.

Finally, the data analysis approach greatly affects MCA's MST performance. Unlike single marker methods, where analysis may be simply contracted out after sample collection, MCA requires collaboration with a research lab having relevant expertise starting with the study design phase. Four laboratories that conducted MCA in the SIPP evaluation study may be good sources of technical advice, including the Holden lab at the University of California Santa Barbara, the Andersen lab at Lawrence Berkeley National Laboratories, Southern California Coastal Water Research Project, and the Sadowsky lab at the University of Minnesota.

CHAPTER 9: SOURCE IDENTIFICATION LIMITATIONS AND ANTICIPATED ADVANCEMENTS IN THE FIELD

This manual provides guidance on best practices for source identification, but some important method limitations remain. This chapter discusses these limitations and the best approaches for minimizing their effects. It also describes scientific investigations currently underway to increase method robustness in the future.

9.1 False Negatives

False negative results (failure to detect a target present in a sample) limit current source tracking technologies the most, since "absence of detection" does not equate to "detection of absence." Sometimes practitioners reach a false negative conclusion because they collect an inadequate number of samples; it takes only a few samples to prove presence of a source, but many more to prove absence. Dilution of source water (e.g., storm drain discharge or ground water) in the surf zone creates concern about detection limits for samples collected at the beach rather than in the watershed.

Beyond those basic sampling design concerns, several method limitations could also lead to a false negative. The most important is qPCR inhibition. Sometimes referred to as interference, inhibition occurs when substances in a sample slow down or block the PCR reaction by interfering with enzymes and/or DNA. Inhibition causes a delayed response in the qPCR reaction, resulting in underestimation of the target DNA or (in some cases) no measurable result. Many common elements and compounds in ambient waters can cause inhibition, such as large organic acids, carbohydrates, and metal cations.

Scientists have developed a number of strategies to address PCR inhibition, several of which, such as DNA purification and use of more robust chemistries, have been incorporated into the SOPs of the recommended methods. In addition, the SOPs (Appendix A) incorporate internal controls to directly assess inhibition in each PCR reaction. When these internal controls indicate an unacceptably high level of inhibition, the most common strategy is to dilute the sample and lower the concentration of inhibitory compounds. While simple and inexpensive, diluting the sample DNA raises detection limits and runs the risk of diluting the signal too much. Scientists are presently working on a new PCR-based technology known as digital PCR, which may virtually eliminate false negatives stemming from inhibition without a concomitant loss in method sensitivity. Such an advance is several years away from routine application.

Another factor, known as target degradation, decay, or aging, may lead to a negative result. Several environmental mechanisms reduce the number of targeted microbes in the water column with distance from the fecal source. These degradation mechanisms include inactivation by sunlight, predation by other organisms, and adsorption to particles and subsequent sedimentation/settling out of the water column. Scientists are beginning to better understand the mechanisms and relative decay rates among indicators. At present, the best strategy to avoid missing a signal due to degradation is to sample as close to the putative source(s) as possible.

9.2 False Positives

In rare circumstances, qPCR assays can produce false positive results, but this concern pales relative to the problem of false negatives because it rarely leads to severe economic consequences (e.g., from remediating a falsely diagnosed source). False positives stem from cross-reactivity, when a positive result for a host-associated microbe comes from a non-target host (e.g., human-associated bacteria found in gull feces). Two types of cross-reactivity occur in human markers. First, the marker may have shortcomings in its specificity. Chapters 5 and 6 generally document these types of cross-reactions, and recommend use of a second confirmatory marker when one or more of the potential cross-reaction sources are present in the watershed.

The second type of cross-reaction occurs when animals, such as domestic pets, live in close association with humans. In some instances, gulls thought to be feeding at landfills containing biosolids from wastewater treatment plants have tested positive for human markers. If you suspect this kind of site-specific cross-reactivity, collect and test for the presence of host-associated markers in fecal samples from animals in the watershed.

9.3 Source Apportionment

Ideally, an MST study would produce a pie chart indicating relative FIB contributions from various host sources, rather than just documenting presence/absence; however, the technology for this type of source allocation does not exist at present. A poor understanding of marker degradation rates creates the greatest difficulty, since sources contributed from different locations in the watershed degrade at different unknown rates before arriving at the sampling site. Scientists are beginning to work on this issue. In the meantime, the best approach is to sample as close as possible to the potential sources based on the hypotheses generated in Step 1 (Chapter 2), rather than relying on multiple markers collected near the base of the watershed.

Even without degradation concerns, scientists are only now beginning to develop mathematical algorithms for how to combine information derived from individual source-specific markers into an integrated assessment. The most promising approach, called the "ratio method," uses information on the ratio of host specific fecal markers and general fecal indicator bacteria in fecal source materials and ambient water samples to deduce the fraction of FIB in an ambient water sample from a specific source. Although the method has worked well for artificial samples created in a laboratory, this method may or may not work on complex field samples. Ongoing research is testing field applications of the ratio method and the effect of aging on its results.

9.4 Source Resolution

While MST methods have advanced significantly in the last decade, researchers continue to improve existing methods and develop promising new technologies and new markers. Sensitive and specific host-associated markers exist for many of the most frequently encountered potential sources, but do not cover all common animals in coastal watersheds (e.g., deer, marine mammals). The technology exists, making marker identification mostly a matter of time.

Increasing the resolution of source specificity represents a bigger advance likely to take place in the next several years. For instance, current human markers do not differentiate between stools from individuals, sewage, or septic sources. Similarly, existing "gull" markers cannot

differentiate between gulls, pigeons, or other seabirds. Microbial community analysis provides higher resolution than qPCR markers, but practitioners could benefit from an even greater level of resolution, such as the ability to differentiate among farms within a watershed.

As scientists continue to test new approaches for MST with many promising results, the potential for better source differentiation, lower costs, and quicker time frames will continue to improve.

FURTHER READING

The following key publications informed the guidance provided in this manual; this section does not constitute a full literature review.

Traditional Methods

Best Management Practices for Sanitary Sewer Overflow (SSO) Reduction Strategies. Central Valley Clean Water Association and Bay Area Clean Water Agencies. 2009. http://bacwa.org/Portals/0/Committees/CollectionSystems/Library/SSO%20BMP%20Manual%201%201.pdf

Existing Sewer Evaluation and Rehabilitation: WEF Manual of Practice No. FD-6 ASCE/EWRI Manuals and Reports on Engineering Practice No. 62, Third Edition, WEF Press: Alexandria, Virginia 2009.

MST Method Evaluation in California

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APPENDIX A: STANDARD OPERATING PROCEDURES (SOPS) FOR RECOMMENDED MST MARKER METHODS

Appendix A1. Membrane filtration for molecular analysis

Equipment and Supplies

Polycarbonate membrane, 47mm, 0.45μm

Manifold setup for filtration (adaptor, vacuum pump and flask etc.)
Filtration funnel (the following protocol uses disposable funnels)
Pre-labeled 2 ml microtube (i.e., bead beating tubes w/ or w/o preloaded beads)
Freezer boxes
Filter forceps
95% ethanol
Beaker
Alcohol lamp or Bunsen burner
Sterile PBS rinsing buffer (pH 7.4, sodium dihydrogen phosphate at 4.085 mM, sodium monhydrogen Phosphate at 20.83 mM, sodium chloride at 145.4 mM in 1L of molecular grade dI water)
Liquid nitrogen and dewar for flash freezing filter tubes (place in dewar before starting filtrations)
Bench recording sheet (operator name, sample name, etc.) and pen
Safety items: gloves, lab coat, safety glasses

Filtration Method

NOTE: Sterilize the bench area thoroughly with 70% ethanol or 10% bleach solution before and after filtration procedure

1. Connect manifold, vacuum tubing, vacuum flasks.
2. Insert adaptor (supplied with funnels) into rubber stopper so it is ready to mount filtration funnels.
3. One should test whether the vacuum assembly is working properly before moving forward. Turn on the pump and close all valves. Then adjust the vacuum pressure using the knurled knob on the vacuum inlet of the pump. (<20 inch Hg, or <0.6 atm).
4. Light the alcohol lamp or Bunsen burner (for flame sterilization).
5. Wear gloves and use aseptic techniques from now on.
6. Place corresponding 2ml microtubes in rack, unscrew cap to loose only (ready for storing membrane filter after completion of filtration).
7. Soak one pair of forceps in a beaker with 95% ethanol.
8. Mount the filtration funnel (i.e. housing) on to the adaptor; label the funnel with sample name.
9. Remove the funnel from the base and place it upside down on the bench on top of its lid.
10. Take one pair of forceps, flame sterilize, allow to cool a little, then pick up the grid filter from the filtration housing, discard the grid filter (be careful not to damage the supporting filter underneath the grid filter).
11. Use the same forceps to carefully pick up one polycarbonate membrane (clear grayish membrane in-between paper separators, do not mistake the paper separator as the

membrane filter), and carefully place it onto the center of the supporting filter (to replace the grid filter discarded in step 10).

12. Put the forceps back into the beaker with ethanol.

13. Mount the filtration funnel (that was set aside in step 9) back onto the housing to secure the polycarbonate membrane (make sure there is no gap between the edge of polycarbonate membrane and the bottom of the filtration funnel and that the edge of the membrane is not folded, i.e. no liquid should go through the housing/support without passing through the polycarbonate membrane first).

14. Take sample bottle off ice, shake and fill the funnel with 100ml water sample (depending on turbidity of the water, one may need to start with a smaller volume), open the vacuum valve and start filtering. Check off on the recording list, or record volume filtered (if filter clogs before designated volume is reached) each time. Rinse the sides of the funnel with 10-20mL PBS once sample volume has gone through.

15. After all liquid passes through the membrane filter, close vacuum valve and remove the funnel.

16. Using a pair of flame sterilized (and cooled) forceps, carefully roll up the polycarbonate membrane filter on the housing. First, fold the membrane in half and hold in place with second forceps. Then, using each pair of forceps alternately, roll the membrane approximately 2 times. Place into the corresponding 2ml microtube (check the label on the tube), screw the cap securely.

17. Flash freeze the filter tube with liquid nitrogen. Keep in liquid nitrogen until samples are ready for transfer to -80ºC freezer (organize the tubes in the freezer box first) and/or extraction.

Appendix A2: DNA EZ ST1 Extraction SOP (GeneRite, LLC)

Equipment and Supplies

Extraction Kit
1.7mL low bind microtubes
10, 100, 1000µL pipets and barrier tips
Kimwipes
DNA Away
Microfuge
Minspin
Vortex
Beadbeater
Microtube rack

Extraction for Filters

NOTE: Lysis buffer can include 10ng/µL salmon DNA stock as a sample processing control (SPC). The salmon DNA is used to determine recovery during extraction. In order to process samples with SPC, add 10µL of salmon stock into 490µL lysis buffer per sample (i.e. if extracting 10 samples, mix 100µL salmon stock and 4900µL lysis buffer together and distribute to each sample tube). Run the Sketa qPCR assay on the samples to measure recovery.

1. Add 500µL (SPC) lysis buffer to each filter tube (i.e. a filter placed into a tube preloaded with beads during filtration process). Do not transfer a frozen filter from one tube to another.
2. Tighten caps and bead beat samples at maximum setting on Bead Beater (biospec) for 2minutes.
3. Centrifuge tubes at 12,000 rcf for 1min.
4. Pipette maximum volume of supernatant out of the bead beating tube, and add to a new 1.5ml microfuge tube. Throughout this extraction do not put tubes next to each other and only have one tube open at a time to minimize the chance of contamination.
5. Centrifuge at 12000 rcf for 1min
6. Pipette out 300µL supernatant (take care not to disturb the pellet) and add it to 1000µL binding buffer in a new, labeled 1.5mL microtube. Pipette up and down to mix and gently vortex.
7. Add 690µL of the DNA/binding buffer mixture (from step 6) to spin column and centrifuge for 1 minute at 10,000 rcf (discard flow through). Place the spin column in new collection tube. Repeat step 7 once.
8. Add 500µL EZ wash buffer and centrifuge for 1 minute at 10,000rcf (discard flow through). Place spin column in new collection tube. Repeat step 8 once.
9. After the second wash, place spin column in another new collection tube and centrifuge at 10,000 rcf for 1min (to get rid of any residual ethanol in the wash buffer).
10. Put column into final 1.7mL low bind microtube. Add 50µL elution buffer, let sit for 1 minute, centrifuge for 1 minute at 10,000 rcf (keep flow through). Repeat step 10 once.

11. Vortex the final 100µL elution buffer/DNA solution and aliquot 50µL or less to other lo-bind microtubes to save for later analysis. The number of aliquots will depend on how many assays will be run with the extract.
12. Run qPCR and/or store extracted DNA in -20°C (non-autodefrost) or -80°C freezer.

Appendix A3: Sketa (Sample Processing Control) qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000μL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer-(SketaF): 5'- GGTTTCCGCAGCTGGG -3' prepared in 500μM stock
Reverse primer-(SketaR): 5'- CCGAGCCGTCCTGGTC-3' prepared in 500μM stock
Probe-(SketaP): [6-FAM]-5'- AGTCGCAGGCGGCCACCGT –TAMRA prepared in 100μM stock
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Salmon DNA (Sigma, D1626)

1. Prepare salmon stocks in a separate area from sample processing and qPCR areas.
2. Create a 10mg/mL stock of the salmon solution by adding salmon DNA to AE buffer and vortexing until solution is homogenous (~4hrs). Note that this DNA material is fibrous and it can take a while to fully dissolve.
3. Quantify the stock solution just created and prepare a 10ug/mL salmon solution by mixing 25μL of 10mg/mL stock into 24.975 mL of AE buffer (just an example concentration). Make 1mL aliquots of this solution for storage at -20°C (need to make new stock every year) or 4°C (need to make new stock every 6 months).

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^1 copies/2μL - 1.00×10^{-3} copies/2μL. This range encompasses the concentration of salmon DNA added to extractions *IF* used for samples. Create the standard curve using a 1:10 dilution series. Run the sketa assay parallel with another target (i.e. HF183, HumM2) to assess extraction recovery. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Primer/Probe Mix Preparation

Create batches of primer probe mix in the reagent hood using dedicated pipets with barrier pipet tips only. Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 500 µM stocks. If probes are dry, reconstitute them in TE (pH 8.0) at 100 µM stocks and create many small aliquots for one time use.

Table 1: Sketa primer/probe mix for simplex applications

Reagent	Stock Solution	Primer/Probe Mix
Forward Primer	500 µM	10 µL
Reverse Primer	500 µM	10 µL
Probe	100 µM	4 µL
PCR clean water	—	576 µL

Master Mix Preparation

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
BSA	2mg/mL	2.5 µL	250 µL
Primer/Probe Mix	1 µM/80 nM	3.0 µL	300 µL
PCR clean water	—	4.0 µL	400 µL

2. Transfer 23 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2µL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load in the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.
7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A4: HF183 Taqman qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000μL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer-(HF-183): 5'- ATCATGAGTTCACATGTCCG -3'
Reverse primer-(BacR287): 5'- CTTCCTCTCAGAACCCCTATCC -3'
TaqMan® probe-(BacP234MGB): [6-FAM]-5'- CTAATGGAACGCATCCC –MGB
TaqMan® probe-(Bac234IAC): [VIC]-5'- AACACGCCGTTGCTACA –MGB
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
HF183Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-cgtcaggtttgtttcggtattgagtatcgaaaatctcacggattaactcttgtgtacgctCTCGAGgaccagctaatg
Catataaataagttacgtgatgagaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctgaaag
gaagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatggggatgcgttccatta
gCTCGAGatagtaggcggggtaacggcccacctagtcaacgatggataggggttctgagaggaagg-3'

IAC Reference (i.e. sequence that can be inserted into a plasmid vector):
5'-atcgcgtcaggtttgtttcggtattgagCCTGCCGTCTCGTGCTCCTCAtctcgaggaccagctaatg
catataaataagttacgtgatgaatgcgaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctga
aaggaagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatgTGTAGCA
ACGGCGTGTTatagtaggcggggtaacggcccacctagtcaacgatggatagggggttctgagaggaagg-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100μL DNase RNase free dI water. Let sit for ~40min. Use 50μL for restriction enzyme digestion, and save the other 50μL for digestion later (you can digest the full 100μL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/μL.
6. Calculate copy/μL using the plasmid size (provided by IDT) and the ng/μL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2μL

7. Prepare stock solution of 10^7copy/2μL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2μL - 1.00×10^1 copies/2μL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve sing a 1:10 dilution series. The IAC plasmid is not used in a standard curve but in the master mix as an internal control to assess inhibition and should amplify at ~32 cycles. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Primer/Probe Mix Preparation

Create batches of primer probe mix in the reagent hood using dedicated pipets with barrier pipet tips only. Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 500 µM stocks. If probes are dry, reconstitute them in TE (pH 8.0) at 100 µM stocks and divide into many small aliquots for one time use.

Table 1: HF183 Taqman primer/probe mix for simplex applications.

Reagent	Stock Solution	Primer/Probe Mix
Forward Primer	500 µM	10 µL
Reverse Primer	500 µM	10 µL
6FAM Probe	100 µM	4 µL
VIC Probe	100 µM	4 µL
PCR clean water	—	572 µL

Master Mix Preparation

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
BSA	2mg/mL	2.5 µL	250 µL
Primer/Probe Mix	1 µM/80 nM	3.0 µL	300 µL
PCR clean water	—	4.0 µL	400 µL
IAC plasmid	—	1 µL	100 µL

2. Transfer 23 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortexed and spin down all DNA templates before inoculation. Add 2µL of template to each well.
5. Cover the inoculated plated with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.
7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A5: HumM2 qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000μL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer-(HumM2F): 5'- CGTCAGGTTTGTTTCGGTATTG -3' prepared as a 500
   μM solution
Reverse primer-(HumM2R): 5'- TCATCACGTAACTTATTTATATGCATTAGC -3'
   prepared as a 500 μM solution
TaqMan® probe-(HumM2P): [6-FAM]-5'- TATCGAAAATCTCACGGATTAACTCTTG
TGTACGC–TAMRA prepared as a 100 μM solution
TaqMan® probe-(UC1P1): [VIC]-5'- CCTGCCGTCTCGTGCTCCTCA –TAMRA
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
HumM2 Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-cgtcaggtttgtttcggtattgagtatcgaaaatctcacggattaactcttgtgtacgctCTCGAGgaccagctaatg
Catataaataagttacgtgatgagaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctgaaag
   gaagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatggggatgcgttccatta
   gCTCGAGatagtaggcggggtaacggcccacctagtcaacgatggatagggttctgagaggaagg-3'

IAC Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-atcgcgtcaggtttgtttcggtattgagCCTGCCGTCTCGTGCTCCTCAtctcgaggaccagctaatg
catataaataagttacgtgatgaatgcgaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctga
   aaggaagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatgTGTAGCA
   ACGGCGTGTTatagtaggcggggtaacggcccacctagtcaacgatggatagggttctgagaggaagg-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100μL DNase RNase free dI water. Let sit for ~40min. Use
   50μL for restriction enzyme digestion, and save the other 50μL for digestion later (you
   can digest the full 100μL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme,
   i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's
   instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance
   reading from above to calculate the plasmid concentration in ng/μL.

6. Calculate copy/μL using the plasmid size (provided by IDT) and the ng/μL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X = total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2μL

7. Prepare stock solution of 10^7 copy/2μL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2μL - 1.00×10^1 copies/2μL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Primer/Probe Mix Preparation

Create batches of primer probe mix in the reagent hood using pipets with barrier pipet tips only. Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 500 μM stocks. Reconstitute probes in TE (pH 8.0) at 100 μM stocks and dispense into many small aliquots for one time use.

Table 1: HumM2 primer/probe mix for simplex applications.

Reagent	Stock Solution	Primer/Probe Mix
Forward Primer	500 μM	10 μL
Reverse Primer	500 μM	10 μL
6FAM Probe	100 μM	4 μL
VIC Probe	100 μM	4 μL
PCR clean water	—	572 μL

Master Mix Preparation

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
BSA	2mg/mL	2.5 µL	250 µL
Primer/Probe Mix	1 µM/80 nM	3.0 µL	300 µL
PCR clean water	—	4.0 µL	400 µL
IAC plasmid	—	1 µL	100 µL

2. Transfer 23 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2µL of template to each well.
5. Cover the inoculated plated with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.
7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol has successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A6: BacCan-UCD qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000µL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer-(BacCan-545f1): 5'- GGAGCGCAGACGGGTTTT -3' prepared as a 100 µM solution
Reverse primer-(BacUni-690r1): 5'- CAATCGGAGTTCTTCGTGATATCTA -3' prepared as a 100 µM solution
Reverse primer-(BacUni-690-r2): 5'- AATCGGAGTTCCTCGTGATATCTA -3' prepared as a 100 µM solution
Probe-(BacUni-656p): [6-FAM]-5'- TGGTGTAGCGGTGAAA -TAMRA–MGB prepared as a 100 µM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
BacCan UCD Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-
-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100µL DNase RNase free dI water. Let sit for ~40min. Use 50µL for restriction enzyme digestion, and save the other 50µL for digestion later (you can digest the full 100µL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/µL.
6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2µL

7. Prepare stock solution of 10^7copy/2µL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2µL - 1.00×10^1 copies/2µL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series . In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Master Mix Preparation

Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 100 µM stocks. Reconstitute probes in TE (pH 8.0) at 100 µM stocks and aliquot for one time use.

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
Forward primer	100 µM	0.1 µL	10 µL
Reverse primer 1	100 µM	0.1 µL	10 µL
Reverse primer 2	100 µM	0.1 µL	10 µL
Probe	100 µM	0.02 µL	2 µL
PCR clean water	—	2.18 µL	218 µL
DNA template	—	10 µL	—

2. Transfer 15 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down All DNA templates before inoculation. Add 10µL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.

7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A7: DogBact qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000µL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer-(DF475F): 5'- CGCTTGTATGTACCGGTACG -3' prepared as a 100 µM solution
Reverse primer-(Bac708R): 5'- CAATCGGAGTTCTTCGTG -3' prepared as a 100 µM solution
Probe-(DogBactP): [6-FAM]-5'- ATTCGTGGTGTAGC GGTGAAATGCTTAG -3' BHQ1 prepared as a 100 µM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):

DogBac Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-
-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100µL DNase RNase free dI water. Let sit for ~40min. Use 50µL for restriction enzyme digestion, and save the other 50µL for digestion later (you can digest the full 100µL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/µL.
6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2μL

7. Prepare stock solution of 10^7copy/2μL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2μL - 1.00×10^1 copies/2μL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) run on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Master Mix Preparation

Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 100 μM stocks. Reconstitute probes in TE (pH 8.0) at 100 μM stocks and aliquot for one time use.

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 μL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 μL	1250 μL
Forward primer	100 μM	0.225 μL	22.50 μL
Reverse primer	100 μM	0.225 μL	22.50 μL
Probe	100 μM	0.075 μL	7.50 μL
PCR clean water	—	9.975 μL	997.5 μL
DNA template	—	2 μL	—

2. Transfer 23 μL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin all DNA templates before inoculation. Add 2μL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.

7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A8: CowM2 qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000μL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer- CowM2F (5'-CGGCCAAATACTCCTGATCGT) prepared as a 500 μM
    solution
Reverse primer- CowM2R (5'-GCTTGTTGCGTTCCTTGAGATAAT) prepared as a 500
    μM solution
Probe- CowM2P ([6-FAM] AGGCACCTATGTCCTTTACCTCATCAACTACAGACA
    [TAMRA]) prepared as a 100 μM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number)
CowM2 Reference (i.e. sequence that can be inserted into a plasmid vector):
5'- CCCGTGTAAA ACGACGGCCA GTTTATCTAG TCAGCTTGAT TCTAGCTGAT
    CGTGGACCGG AAGGTGAGCC AGTGAGTTGATTGCAGTCCA GTTACGCTGG
    AGTCTGAGGC TCGTCCTGAA TGATATGCGA CCGCCGGAGG GTTGCGTTTG
    AGACGGGCGACAGATCGACA CTGCTCGATC CGCTCGCACC CGATCGATGG
    CATAGTTCTT CCGCATGGTA GATCATGAGT TCACATGTCCGGGATAATTA
    TTAAAGAATT TCGGTTGTCG ATGGGGATGC GTTCCATTAG GCAGTTGGCG
    GGGTAACGGC CCACTCGAGCTACGATGGAT AGGGGTTCTG AGAGGAAGGT
    CCCCCACATT GGAACTGAGA CACGGTCCAA ACTCCTACGG
    GAGCGTCAGGTTTGTTTCGG TATTGAGTAT CGAAAATCTC ACGGATTAAC
    TCTTGTGTAC GCTCTCGAGG ACCAGCTAAT GCATATAAATAAGTTACGTG
    ATGACGGCCA AATACTCCTG ATCGTACTCG AGATAGGCAC CTATGTCCTT
    TACCTCATCA ACTACAGACAAAATTATCTC AAGGAACGCA ACAAGCCCTC
    TAATGGAAAA TGGATGGTAT CTTTGGAGCC TTTGAAAGCA
    CTCGAGCCTTATGCATTGAG CATCGAGGCC GGAAAGCAGG AACTTATATA
    TAATAAGGTA TTAGCAGGCG AAGTATGGAT GGCTTGCTAAATTTGATGGC
    GACCGGCGCA CGGGATCCTA ACGCGTATCC AACCTTCCCT TATCCACGGG
    ATAGCCCGTC GAAAGGCGGATTAATACCGT ATGAGGTGCG GCCGCGGATC
    GACGAGAGCA GCGCGACTGG ATCAGTTCTG GACGAGCGAG
    CTGTCGTCCGACCCGTGATC TTACGGCATT ATACGTATGA TCGGTCCACG
    ATCAGCTAGA TTATCTAGTC AGCTTGATGT CATAGCTGTTTCCTGAGGCT
    CAATACTGAC CATTTAAATC ATACCTGACC TCCATAGCAG AAAGTCAAAA
    GCCTCCGACC GGAGGCTTTTGACTTGATCG GCACGTAAGA GGTTCCAACT

TTCACCATAA TGAAATAAGA TCACTACCGG GCGTATTTTT
TGAGTTATCGAGATTTTCAG GAGCTAAGGA AGCTAAAATG AGTATTCAAC
ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTGCCTTCCTGTT
TTTGCTCACC CAGAAACGCT GGTGAAAGTA AAAGATGCTG AAGATCAGTT
GGGTGCACGA GTGGGTTACATCGAACTGGA TCTCAACAGC GGTAAGATCC
TTGAGAGTTT ACGCCCCGAA GAACGTTTTC CAATGATGAG
CACTTTTAAAGTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG
GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAATGACTTGGTT
GAGTACTCAC CAGTCACAGA AAAGCATCTC ACGGATGGCA TGACAGTAAG
AGAATTATGC AGTGCTGCCATAACCATGAG TGATAACACT GCGGCCAACT
TACTTCTGGC AACGATCGGA GGACCGAAGG AGCTAACCGC
TTTTTTGCACAACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC
CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACACCACGATGCCT
GTAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC
TCTAGCTTCC CGGCAACAATTAATAGACTG GATGGAGGCG GATAAAGTTG
CAGGATCACT TCTGCGCTCG GCCCTCCCGG CTGGCTGGTT
TATTGCTGATAAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG
CAGCACTGGG GCCAGATGGT AAGCCCTCCC GCATCGTAGTTATCTACACG
ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT
AGGTGCCTCA CTGATTAAGCATTGGTAATG AGGGCCCAAA TGTAATCACC
TGGCTCACCT TCGGGTGGGC CTTTCTTGAG GACCTAAATG
TAATCACCTGGCTCACCTTC GGGTGGGCCT TTCTGCGTTG CTGGCGTTTT
TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCGATGCTCAAGT
CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC
TGGAAGCTCC CTCGTGCGCTCTCCTGTTCC GACCCTGCCG CTTACCGGAT
ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC
TCATAGCTCACGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA
AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCGCTGCGCCTTA
TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC
ACTGGCAGCA GCCACTGGTAACAGGATTAG CAGAGCGAGG TATGTAGGCG
GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA
CACTAGAAGAACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT
CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACCACCGCTGGTA
GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG AAAAAAAGGA
TCTCAAGAAG ATCCTTTGATTTTCTACCGA AGAAAGGCCC A-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100μL DNase RNase free dI water. Let sit for ~40min. Use 50μL for restriction enzyme digestion, and save the other 50μL for digestion later (you can digest the full 100μL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/μL.

6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2µL

7. Prepare stock solution of 10^7 copy/2µL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2µL - 1.00×10^1 copies/2µL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Primer/Probe Mix Preparation

Create batches of primer probe mix in the reagent hood using dedicated pipets with barrier pipet tips only. Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 500 µM stocks. Reconstitute probes in TE (pH 8.0) at 100 µM stocks and aliquot for one time use.

Table 1: CowM2 primer/probe mix for simplex applications.

Reagent	Stock Solution	Primer/Probe Mix
Forward Primer	500 µM	10 µL
Reverse Primer	500 µM	10 µL
Probe	100 µM	4 µL
PCR clean water	—	576 µL

Master Mix Preparation

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
BSA	2mg/mL	2.5 µL	250 µL
Primer/Probe Mix	3.5µL	3.5 µL	350 µL
PCR clean water	—	4.5 µL	450 µL
DNA template	—	2 µL	—

2. Transfer 23 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2µL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.
7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run.
9. After the run, applying the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A9: Rum2Bac qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000µL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer- BacB2-590F (5'- ACAGCCCGCGATTGATACTGGTAA) prepared as a
500 µM solution
Reverse primer- Bac708Rm (5'- CAATCGGAGTTCTTCGTGAT) prepared as a 500 µM
solution
Probe- BacB2-626P ([6-FAM] ATGAGGTGGATGGAATTCGTGGTGT [BHQ1]) prepared
as a 100 µM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
Rum2Bac Reference (i.e. sequence that can be inserted into a plasmid vector)
5'- -3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100µL DNase RNase free dI water. Let sit for ~40min. Use
   50µL for restriction enzyme digestion, and save the other 50µL for digestion later (you
   can digest the full 100µL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme,
   i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's
   instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance
   reading from above to calculate the plasmid concentration in ng/µL.
6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration
   from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2µL

7. Prepare stock solution of 10^7 copy/2µL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2µL - 1.00×10^1 copies/2µL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Primer/Probe Mix Preparation

Create batches of primer probe mix in the reagent hood using pipets with barrier pipet tips only. Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 500 µM stocks. Reconstitute probes in TE (pH 8.0) at 100 µM stocks and aliquot for one time use.

Table 1: CowM2 primer/probe mix for simplex applications.

Reagent	Stock Solution	Primer/Probe Mix
Forward Primer	500 µM	10 µL
Reverse Primer	500 µM	10 µL
Probe	100 µM	4 µL
PCR clean water	—	576 µL

Master Mix Preparation

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
BSA	2mg/mL	2.5 µL	250 µL
Primer/Probe Mix	3.5µL	3.5 µL	350 µL
PCR clean water	—	4.5 µL	450 µL
DNA template	—	2 µL	—

2. Transfer 23 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2µL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.
7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run.
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.

Appendix A10: Pig2Bac qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000μL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer- Pig-2-Bac41F (5'- GCATGAATTTAGCTTGCTAAATTTGAT -3')
    prepared as a 500 μM solution
Reverse primer- Pig-2-Bac 163Rm (5'- ACCTCATACGGTATTAATCCGC -3') prepared as
    a 500 μM solution
Fluorigenic probe- Pig-2-Bac 113P ([6-FAM] TCCACGGGATAGCC [MGB]) prepared as a
    100 μM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
Pig Reference (i.e. sequence that can be inserted into a plasmid vector)
5'- CCCGTGTAAA ACGACGGCCA GTTTATCTAG TCAGCTTGAT TCTAGCTGAT
    CGTGGACCGG AAGGTGAGCC AGTGAGTTGATTGCAGTCCA GTTACGCTGG
    AGTCTGAGGC TCGTCCTGAA TGATATGCGA CCGCCGGAGG GTTGCGTTTG
    AGACGGGCGACAGATCGACA CTGCTCGATC CGCTCGCACC CGATCGATGG
    CATAGTTCTT CCGCATGGTA GATCATGAGT TCACATGTCCGGGATAATTA
    TTAAAGAATT TCGGTTGTCG ATGGGGATGC GTTCCATTAG GCAGTTGGCG
    GGGTAACGGC CCACTCGAGCTACGATGGAT AGGGGTTCTG AGAGGAAGGT
    CCCCCACATT GGAACTGAGA CACGGTCCAA ACTCCTACGG
    GAGCGTCAGGTTTGTTTCGG TATTGAGTAT CGAAAATCTC ACGGATTAAC
    TCTTGTGTAC GCTCTCGAGG ACCAGCTAAT GCATATAAATAAGTTACGTG
    ATGACGGCCA AATACTCCTG ATCGTACTCG AGATAGGCAC CTATGTCCTT
    TACCTCATCA ACTACAGACAAAATTATCTC AAGGAACGCA ACAAGCCCTC
    TAATGGAAAA TGGATGGTAT CTTTGGAGCC TTTGAAAGCA
    CTCGAGCCTTATGCATTGAG CATCGAGGCC GGAAAGCAGG AACTTATATA
    TAATAAGGTA TTAGCAGGCG AAGTATGGAT GGCTTGCTAAATTTGATGGC
    GACCGGCGCA CGGGATCCTA ACGCGTATCC AACCTTCCCT TATCCACGGG
    ATAGCCCGTC GAAAGGCGGATTAATACCGT ATGAGGTGCG GCCGCGGATC
    GACGAGAGCA GCGCGACTGG ATCAGTTCTG GACGAGCGAG
    CTGTCGTCCGACCCGTGATC TTACGGCATT ATACGTATGA TCGGTCCACG
    ATCAGCTAGA TTATCTAGTC AGCTTGATGT CATAGCTGTTTCCTGAGGCT
    CAATACTGAC CATTTAAATC ATACCTGACC TCCATAGCAG AAAGTCAAAA
    GCCTCCGACC GGAGGCTTTTGACTTGATCG GCACGTAAGA GGTTCCAACT

TTCACCATAA TGAAATAAGA TCACTACCGG GCGTATTTTT
TGAGTTATCGAGATTTTCAG GAGCTAAGGA AGCTAAAATG AGTATTCAAC
ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTGCCTTCCTGTT
TTTGCTCACC CAGAAACGCT GGTGAAAGTA AAAGATGCTG AAGATCAGTT
GGGTGCACGA GTGGGTTACATCGAACTGGA TCTCAACAGC GGTAAGATCC
TTGAGAGTTT ACGCCCCGAA GAACGTTTTC CAATGATGAG
CACTTTTAAAGTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG
GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAATGACTTGGTT
GAGTACTCAC CAGTCACAGA AAAGCATCTC ACGGATGGCA TGACAGTAAG
AGAATTATGC AGTGCTGCCATAACCATGAG TGATAACACT GCGGCCAACT
TACTTCTGGC AACGATCGGA GGACCGAAGG AGCTAACCGC
TTTTTTGCACAACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC
CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACACCACGATGCCT
GTAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC
TCTAGCTTCC CGGCAACAATTAATAGACTG GATGGAGGCG GATAAAGTTG
CAGGATCACT TCTGCGCTCG GCCCTCCCGG CTGGCTGGTT
TATTGCTGATAAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG
CAGCACTGGG GCCAGATGGT AAGCCCTCCC GCATCGTAGTTATCTACACG
ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT
AGGTGCCTCA CTGATTAAGCATTGGTAATG AGGGCCCAAA TGTAATCACC
TGGCTCACCT TCGGGTGGGC CTTTCTTGAG GACCTAAATG
TAATCACCTGGCTCACCTTC GGGTGGGCCT TTCTGCGTTG CTGGCGTTTT
TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCGATGCTCAAGT
CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC
TGGAAGCTCC CTCGTGCGCTCTCCTGTTCC GACCCTGCCG CTTACCGGAT
ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC
TCATAGCTCACGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA
AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCGCTGCGCCTTA
TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC
ACTGGCAGCA GCCACTGGTAACAGGATTAG CAGAGCGAGG TATGTAGGCG
GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA
CACTAGAAGAACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT
CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACCACCGCTGGTA
GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG AAAAAAAGGA
TCTCAAGAAG ATCCTTTGATTTTCTACCGA AGAAAGGCCC A-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100μL DNase RNase free dI water. Let sit for ~40min. Use 50μL for restriction enzyme digestion, and save the other 50μL for digestion later (you can digest the full 100μL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/μL.

6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2µL

7. Prepare stock solution of 10^7copy/2µL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2µL - 1.00×10^1 copies/2µL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Primer/Probe Mix Preparation

Create batches of primer probe mix in the reagent hood using dedicated pipets with barrier pipet tips only. Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 500 µM stocks. Reconstitute dry probes in TE (pH 8.0) at 100 µM stocks and aliquot for one time use.

Table 1: Pig-2-Bac primer/probe mix for simplex applications.

Reagent	Stock Solution	Primer/Probe Mix
Forward Primer	500 µM	10 µL
Reverse Primer	500 µM	10 µL
Probe	100 µM	4 µL
PCR clean water	—	576 µL

Master Mix Preparation

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 µL	1250 µL
BSA	2mg/mL	2.5 µL	250 µL
Primer/Probe Mix	3.5µL	3.5 µL	350 µL
PCR clean water	—	4.5 µL	450 µL
DNA template	—	2 µL	—

2. Transfer 23 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2µL of template to each well.
5. Cover the inoculated plated with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.
7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Export the data for analysis.

Appendix A11: Horse Conventional PCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000µL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
Flash gel cassettes, camera, DNA ladder and dye (VWR Lonza starter kit, catalogue # 95015-612)
Voltage source
Takara Ex Taq Kit (Takara, Catalogue # RR001AM)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer- HoF597 (5'- CCA GCC GTA AAA TAG TCG G -3') prepared as a 10 µM solution
Reverse primer- Bac708R (5'- CAA TCG GAG TTC TTC GTG -3') prepared as a 10 µM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
Horse Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-
-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100µL DNase RNase free dI water. Let sit for ~40min. Use 50µL for restriction enzyme digestion, and save the other 50µL for digestion later (you can digest the full 100µL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/µL.
6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2μL

7. Prepare stock solution of 10^7copy/2μL based on the concentration above, and aliquot in small volumes for future use.

Use the plasmid stock solution prepared above as a positive control. In addition, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Master Mix Preparation

Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 100 μM stocks. Reconstitute probes TE (pH 8.0) at 100 μM stocks and aliquot for one time use.

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 μL Reaction	Master Mix (100 rxns)
Takara ExTaq PCR buffer (no MgCl$_2$)	10X	2.5 μL	250 μL
MgCl$_2$	25 mM	2.0 μL	200 μL
Takara ExTaq dNTPs (2.5 mM each)	2500 μM	2.0 μL	200 μL
Forward primer	10 μM	0.5 μL	50 μL
Reverse primer	10 μM	0.5 μL	50 μL
BSA	2 mg/mL	0.25 μL	25 μL
Takara ExTaq DNA Polymerase	5 U/μL	0.125 μL	12.5 μL
PCR clean water	—	15.125 μL	1512.5 μL
DNA template	—	2 μL	—

2. Transfer 23 μL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2μL of template to each well.
5. Cover the inoculated plated with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software. Note: It is important that a plate layout is created (in Excel/Lab Notebook) in order to keep track of which samples are running.

7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	94	2 min
35	94	45 sec
35	61	45 sec
35	72	45 sec
1	72	7 min

8. Once the run is completed, store PCR products at 4°C until visualization on a gel, ideally within 24hrs of the PCR run. Use a double tier Flash Gel cassette. Mix 2 µL of PCR product with 2µL of 1X loading dye. Load all 4µL into the gel and run at 270 volts for 4 min. Take multiple pictures. Note: Traditional gels can be made and used for visualization as well.

Appendix A12: Gull2 Taqman qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000μL pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer- Gull2 F (5'- TGCATCGACCTAAAGTTTTGAG -3') prepared as a 100
    μM solution
Reverse primer- Gull2 R (5'- GTCAAAGAGCGAGCAGTTACTA -3') prepared as a 100
    μM solution
Fluorigenic probe- Gull2 P ([6-FAM] CTGAGAGGGTGATCGGCCACATTGGGACT
    [BHQ1]) prepared as a 100 μM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):
Gull Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-
-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100μL DNase RNase free dI water. Let sit for ~40min. Use 50μL for restriction enzyme digestion, and save the other 50μL for digestion later (you can digest the full 100μL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/μL.
6. Calculate copy/μL using the plasmid size (provided by IDT) and the ng/μL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2μL

7. Prepare stock solution of 10^7copy/2μL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2μL - 1.00×10^1 copies/2μL. This range can be adjusted to encompass the most appropriate concentrations of target. Create the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Master Mix Preparation

Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 100 μM stocks. Reconstitute probes in TE (pH 8.0) at 100 μM stocks and aliquot for one time use.

Prepare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 μL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	12.5 μL	1250 μL
Forward primer	100 μM	0.23 μL	23 μL
Reverse primer	100 μM	0.23 μL	23 μL
Probe	100 μM	0.008 μL	0.8 μL
PCR clean water	—	5.25 μL	525 μL
DNA template	—	2 μL	—

2. Transfer 23 μL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down all DNA templates before inoculation. Add 2μL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.

7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
40	95	15 sec
40	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported.

Appendix A13: Lee Seagull qPCR SOP

Equipment and Supplies

1.7mL low bind microtubes
10, 100, 1000µM pipets and barrier tips
Kimwipes
DNA Away
10% Bleach
Minspin
Vortex
Microtube rack
Laminar flow hoods (one for 'reagents only' and one for 'DNA only')
Thermal Cycler
TaqMan Environmental PCR Master Mix (Applied Biosystems, Catalogue # 4396838)
2 mg/mL Bovine serum albumen (BSA), fraction V
Forward primer- CaT#998F (5'- AGGTGCTAATACCGCATAATACAGAG -3') prepared as a 10 µM solution
Reverse primer- CaT#998R (5'- GCCGTTACCTCACCGTCTA-3') prepared as a 10 µM solution
Fluorigenic probe- CaT#998P ([6-FAM] TTCTCTGTTGAAAGGCGCTT [MGB]) prepared as a 10 µM solution
AE buffer (Qiagen #19077)
Ultrapure water (Specialty Media, Phillipsburg, NJ #TMS-006-A)
Plasmid containing target (at known copy number):

Gull Reference (i.e. sequence that can be inserted into a plasmid vector)
5'-
-3'

1. Prepare standards in a separate area from sample processing and qPCR areas.
2. In the tube from IDT add 100µL DNase RNase free dI water. Let sit for ~40min. Use 50µL for restriction enzyme digestion, and save the other 50µL for digestion later (you can digest the full 100µL in one batch if needed).
3. Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4. Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5. Measure the final product's A260 absorbance reading in triplicate. Use the absorbance reading from above to calculate the plasmid concentration in ng/µL.
6. Calculate copy/µL using the plasmid size (provided by IDT) and the ng/µL concentration from above:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(X \text{ bp})(650 \text{ Daltons/bp})} = Y \text{ plasmid molecule molecules/gram}$$

X=total # of bps in plasmid

Use the absorbance to calculate the concentration of the plasmid in copies/2µL

7. Prepare stock solution of 10^7copy/2µL based on the concentration above, and aliquot in small volumes for future use.

Prepare standard curves from the plasmid stock solution prepared above. The curve should range from 1.00×10^6 copies/2µL - 1.00×10^1 copies/2µL. This range can be adjusted to encompass the most appropriate concentrations of target. Creating the standard curve using a 1:10 dilution series. In addition to a standard curve, run negative extraction controls (NEC) and No template controls (NTC) on each plate.

NOTE: Use a dedicated Laminar flow hood containing all needed equipment when preparing master mixes for qPCR. Wipe surfaces with 10% bleach solution and let sit for 10 minutes before wiping away. Additionally, apply UV light to all hoods before and after use for at least 30 minutes. If possible prepare DNA template in a hood separate from where reagents are prepared to avoid contamination. Always use disposable gloves and wear a lab coat.

Master Mix Preparation

Reconstitute dried primers with AE buffer as recommended by the manufacturer to create 100 µM stocks. Reconstitute probes in TE (pH 8.0) at 100 µM stocks and aliquot for one time use.

Preapare reagents in dedicated 'Reagent only' Laminar flow hood using dedicated laboratory equipment and supplies.

1. Prepare respective MasterMix preparation as described below:

Reagent	Stock Solution	25 µL Reaction	Master Mix (100 rxns)
Environmental Master Mix	2X	10.0 µL	1000 µL
Forward primer	10 µM	0.63 µL	62.5 µL
Reverse primer	10 µM	0.63 µL	62.5 µL
Probe	10 µM	0.31 µL	31.25 µL
PCR clean water	—	3.44 µL	344.0 µL
DNA template	—	5 µL	—

2. Transfer 15 µL of master mix into each 96-well plate.
3. Cover tray with aluminum adhesive tape, label, and store in dark on ice for transport to 'DNA only' hood for template inoculation.
4. Vortex and spin down All DNA templates before inoculation. Add 5µL of template to each well.
5. Cover the inoculated plate with PCR sealing film and load onto the thermal cycler.
6. Launch the thermal cycling software and create a plate setup. Clearly label each sample and the controls.

7. The thermal cycling protocol is as follows:

Cycle Number	Temperature (°C)	Time
1	50	2 min
1	95	10 min
45	95	15 sec
45	60	1 min

8. After the protocol is successfully loaded, start the run
9. After the run, apply the correct baseline to the data (may vary from platform to platform, i.e. 0.08 on ABI, 100 on CFX). Data can then be exported for analysis.