FINAL REPORT

DNR Project 229

Field Verification of Adenovirus Assays for Source Tracking

Prepared by:

Sharon C. Long, Jessie W. Dowding, Hannah Zeeb, Terra Hawley, Tinamarie Schmansky, and Zachary Tracy

May 14, 2018

Executive Summary

Individual qPCR assays for bovine adenovirus and porcine adenovirus targets were adapted from assays published in the literature for use with TaqMan chemistry on the ABI 7500 platform. The intent was to add these assays to the State of Wisconsin's toolbox for source investigations of drinking water contamination. The performance of these assays were examined under various sample concentration and clean-up approaches. The optimized assays were used to test different sources (feces and wastewater) and environmental samples for specificity, sensitivity and cross-reactivity. In addition, initial experimentation was conducted to evaluate the potential of multi-plexing these two assays into one. Overall, the workflow and assays are summarized in the *Conclusions and Recommendations* section of this report. As with all microbial source tracking (MST) assays, supplemental information such as sanitary surveys and careful interpretation of assay results are also key to their effective use.

Introduction

Safe drinking water remains a concern in developed nations despite improved treatment technologies and stringent source water and wellhead protection standards. The Clean Water Act of 1972 effectively reduced water pollution through widespread application of wastewater treatment. However, since the origins of fecal pollution to source waters is diverse and varied, the problem has not been completely eliminated (Presidents Clean Water Action Plan, 1998). The 1986 Amendments to the Safe Drinking Water Act established the national Wellhead Protection Program. Watershed and wellhead protection focuses on reducing the impact of human activities on source waters ensuring that they meet criteria for their designated use. Approximately half of Americans and 95 percent of rural Americans obtain their drinking water from groundwater (U.S. EPA, 2006). In Wisconsin, farming of over 1.2 million dairy cows, a sizeable pork industry and growing populations of feral pigs results in a great potential for contamination of groundwater from manure storage and land spreading.

The need to protect watersheds from fecal contamination has led to an investigation of better monitoring tools. A suite or toolbox of tests that have been developed to supplement monitoring for indicator organisms is called "Fecal Source Tracking" (FST). Microbial source tracking (MST) is a subset of this toolbox that focuses specifically on microbial targets that have been shown to be more or less associated with one particular species of animal or animal group (for example grazing animals, domesticated animals, or avian species). A number of bacterial MST

tools have been in use at the Wisconsin State Laboratory of Hygiene (WSLH) for investigative and enforcement samples. These include: *Rhodococcus coprophilus*, sorbitol-fermenting *Bifidobacteria*, human adenovirus and various *Bacteroides* species. *Rhodococcus coprophilus* has been demonstrated to identify the specific presence of grazing animal fecal matter, manure, in contaminated waters (Rowbotham and Cross 1977; Mara and Oragui, 1981; Mara and Oragui 1983; Savill *et al.*, 2001; Plummer and Long, 2009). Sorbitol-fermenting *Bifidobacteria* have been associated with human feces and domestic sewage (Long *et al.*, 2005). Other *Bifidobacteria* species have recently also been associated with bovine, swine and poultry wastes, respectively (Gòmez-Donaté *et al.*, 2012). While different *Bacteroides* species have been associated with human or bovine or ruminant wastes (Layton *et al.*, 2006; Kildare *et al.*, 2007; Shanks *et al.*, 2008 and 2009; Green *et al.*, 2014). Human adenovirus has been demonstrated to be strongly associated with domestic wastewater (Plummer and Long, 2013). However, recent work in the PI's laboratory has demonstrated that there is a significant proportion of cross reaction for the bacterial targets (Long *et al.*, 2015). This makes a single positive result conclusive for fecal contamination, but equivocal as to a specific source. Therefore, consistent with scientific consensus, an integrated approach incorporating sanitary survey and land use data is needed. In addition, more accurate MST tools are also needed.

Adenoviruses (AdV) have been suggested as potentially valuable, source-specific fecal indicator organisms for livestock as well as humans. One MST test that has demonstrated no cross reactivity in testing with over 100 samples from across the US as well as across Wisconsin is the Human Adenovirus quantitative polymerase chain reaction (qPCR) assay. In a prior project (WR09R002), the PI as part of another research team assisted in developing methods for the concentration and discrimination of bovine AdV in water samples, adding this valuable MST target to the "toolbox" of methods available to the Wisconsin State Laboratory of Hygiene. However, additional work is required to expand the lab's virus-detection capabilities and to validate and optimize viral MST assays. The objectives of the research presented here were to: verify qPCR methods for the quantification of bovine AdV; adapt an assay for porcine AdV; determine the prevalence and genomic signature of these AdV in Wisconsin bovine and swine manure samples; evaluate the cross reaction among numerous other animal manure samples; and verify the presence of various AdV in selected Revised Total Coliform Rule (RTCR) unsafe wells to elucidate the value of these assays as MST tools for the State of Wisconsin.

With over 1.2 million dairy cows in Wisconsin as well as swine production and growing feral pig populations, the ability to track bovine and porcine fecal contamination separate from other agricultural animals would greatly improve wellhead protection programs. With improved detection methods, bovine and porcine adenovirus assays can become an easily applied and valuable MST tool for watershed and wellhead protection programs applied to Department of Natural Resources and Department of Health Services samples, among others.

Steps in verifying and developing a qPCR-based method include optimizing: (1) sample concentration, (2) extraction and purification of nucleic acid, (3) PCR amplification, (4) confirmation of presence of target amplicon, (5) quantification of nucleic acid concentration against a standard curve, and (6) verification with environmental samples. Various sample concentration techniques, nucleic acid purification methods, and PCR approaches were explored. To evaluate the performance of the resulting PCR-based method using environmental samples, results were compared against the results of indicator organism culture-based analyses (total coliform, *E. coli,* enterococci, and API 20E speciation) and MST PCR-based analyses (*R. coprophilus, Bifidobacteria* species, *Bacteroides* species, and human Adenovirus) being conducted under the Revised Total Coliform Rule unsafe well assessment program. This work leverages the large volume sampling program initiated using a previous Groundwater Coordinating Council grant (DNR Project #222). These additional AdV assays can be added to the WSLH toolbox for investigating sources of contamination to drinking water.

Methods and Approach

Sample Concentration

Unlike bacterial targets, adenoviruses are too small to be captured on 0.45 µm membrane filters. Hollow fiber ultrafiltration has been demonstrated to effectively concentrate particles as small as 0.01 µm and has been well studied for concentration of various microbes in water (Sobsey and Simmons, 2009). Virus researchers have suggested that PEG precipitation of environmental samples to be an efficient method for virus concentration (Rådström *et al.,* 2004; Polaczyk *et al.,* 2008; Sibley, 2009). Alternatively, ultracentrifugation is effective at concentrating particulates including colloids such as viruses in aqueous samples. However, because of the requirements for expensive, non-standard laboratory equipment and safety considerations, it was determined that PEG precipitation would potentially be more transferable to other environmental laboratories.

In previous studies by the PI, polyethylene glycol (PEG) precipitation has been used as a secondary concentration method for HFUF concentrates of well samples (Plummer and Long, 2013). Briefly, 10 to 100 liters of water are concentrated to approximately 1 liter by HFUF. Then, 200 mL of HFUF concentrate is amended with beef extract (2% w/v), sodium chloride (0.3 M), and polyethylene glycol 8000 (10% w/v). The suspension is adjusted to between pH 7.2 and 7.4 and shaken overnight at 4°. After a series of centrifugation steps, the viruses are concentrated in a pellet of up to 1mL in volume which can then be subject to nucleic acid extraction and purification. Verification of the efficiency of performance of this step of the method was evaluated by analyzing synthetic samples prepared using AdV positive bovine and porcine manure samples. Described more fully under *Quantification*, PEG pellets were spiked with known quantities of target DNA (GBlocks) and assayed for recovery efficiency in various experiments. Factors that can be manipulated to improve this step, are final concentrations of the precipitate mix, and centrifuge times and speeds. The recommended standard operating procedures (SOPs) for HFUF concentration and PEG precipitation are in the report Appendix.

Extraction and Purification of Nucleic Acid

Traditional methods for separating environmental compounds that inhibit the PCR reaction (organic matter and metals) include sephadex column clean-up and alcohol precipitation/resuspension and potential use of highly toxic compounds, guanidinium thiocyanate. These methods are labor intensive and are difficult to standardize for use in production laboratory settings. Recently, many commercial nucleic acid extraction and clean-up kits have become commonly applied to environmental samples having been proven effective in the clinical setting. Therefore, commercial products were investigated by the PI's research group based on commercial literature, discussions with company technical representatives, and prior experience in the PI's group. The MoBio PowerSoil DNA Isolation kit (Carlsbad, CA) extraction was determined to provide the most quantitative and amplifiable DNA for virus qPCR assays for the amount of labor involved among five methods that were compared for the target Torque Teno Virus (Plummer and Long, 2013). Verification of the efficiency of performance of this method was evaluated by analyzing synthetic samples prepared using AdV positive bovine and porcine manure samples. This step in the overall sample processing procedure was also evaluated by using GBlock spikes. One key factor that was investigated was the potential use of both the MoBio PowerMax Soil DNA Isolation kit and Zymo DNA Isolation kit (Irvine, CA) was the

ability for the extraction of larger volumes of material into 2 to 5 mL final volume. The recommended extraction and purification methods are the Zymo DNA Isolation kit for PEG pellets and the MoBio PowerMax Soil DNA Isolation kit for fecal samples (see *Results* below). The recommended SOPs for extraction and purification are contained in the Appendix of this report.

PCR Amplification

The quantitative PCR assays chosen for evaluation are based upon research by Pedersen *et al.,* 2011 and Hundesa 2009 for bovine and porcine AdV, respectively. A summary of the assays are presented in Table 1. Although these assays have been optimized by the previous researchers, the project used an ABI 7500 Fast platform; recommended by the US EPA for analyses of indicator organisms in Clean Water Act samples. The previous assays were optimized using Roche and Stratagene platforms, respectively. Assay performance can vary from platform to platform. Factors that can affect the performance of these assays on the ABI platform include Master mix composition, primer and probe concentrations, temperature ramping speed, and sensitivity of optical detectors. The first two can be empirically manipulated while the latter two are a function of the PCR platform.

Table 1. Anticipated qPCR Summaries

Target	Primers and Probes	Thermocycling Conditions
Bovine Adenovirus (BAdV10) (127 base pairs)	Forward B10F: TTACGCCCAACTTCCTTTTG Reverse B10R: CCACGCGTCTACTCCGTATT 127 Probe: SYBR green assay	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 58°C 40 sec, 72° C 1 sec; 60° C 60 sec
Porcine Adenovirus (PAdV) (68 base pairs)	Forward Q-PAdV-F: AACGGCCGCTACTGCAAG Reverse Q-PAdV-R: GCAGCAGGCTCTTGAGG Probe Q-PAdV-P: FAM-CACATCCAGGTGCCGC-BHQ1	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 55°C 20 sec; 60° 20 sec

Review of current literature revealed that not all *Taq* performs equivalently. Rådström *et al*. (2004) note that DNA polymerase can be negatively affected by compounds within biological samples, and that the choice of DNA polymerase can influence the performance of several PCR-based applications. The authors emphasize the importance of choosing the right DNA polymerase for the intended application, noting that commercially available *Taq* polymerase can be inhibited by humic substances (Rådström *et al.,* 2004). Based on previous experience in the PI's laboratory, the use of Life Technologies Environmental MasterMix 2.0 provides the best *Taq* performance in the presence of environmental inhibitors. A minimal number of experiments using products such as HotStar Taq (Qiagen, Duesseldorf, Germany) and AmpliTaq Gold Master mix (Applied Biosystems Inc., Foster City, CA) were conducted in previous research to confirm the efficacy of Master mix performance (Plummer and Long, 2013).

While primer and probe concentrations in the published assays were optimized for use on different platforms. Typically, concentrations between 200 and 600 nM are used in the qPCR assay. However, in certain virus assays, probe concentrations as high as 900 nM were required for optimal detection (Plummer and Long, 2013). Therefore, quantities of known DNA target were tested with different primer and probe concentration combinations and qPCR efficiencies

were determined. Those combinations of concentrations that provided the highest efficiency (E between 90 and 105 percent) and lowest level of quantification (LOQ) will be chosen for the final assay. The final concentrations of primers and probes and the optimized thermocycling programs are summarized in the MasterMix Templates for each assay, contained in the Appendix of this report.

Finally, a selected number of experiments were conducted to determine whether these assays can be conducted in single-plex or multi-plex with each other or with the human AdV assay. Changes in precision among duplicates and efficiency of each primer set when multi-plexed determine the answer to this question. The ability to multi-plex assays would improve sample throughput resulting in shorter turn around times for data delivery. The results of this comparison is presented in the *Results* section of this report. While some efficiency and sensitivity in the assay is lost through multi-plex analyses, for urgent situations, multi-plex analyses can be applied with critical analyses of the results. The MasterMix Template for this combined assay is contained in the Appendix of this report.

Confirmation of Target Amplicon

Since pathogenic viruses often circulate within a single species population and mostly do not jump host species, a viral indicator that is unique to bovine or porcine hosts would be a desirable characteristic. One assessment of whether the target amplified sequence was highly bovine-specific or porcine-specific would be to conduct an *in silico* analysis. In the work conducted by Pedersen *et al.,* 2011 and Hundesa 2009, amplified sequences were subject to sequence alignment in the National Center for Biotechnology Information (NCBI) BLAST utility. These analyses confirm the theoretical specificity of the assay. Analyses of source samples (sewage, animal feces, animal urine, and manure lagoon/slurries) also helps to confirm the extent of non-specific amplification or cross-infection that occurs. An evaluation of a suite of source samples is presented in the *Results* section of this report.

Quantification of Nucleic Acids

While the chemistries used in qPCR can be unstable, currently the best ways to obtain a quantitative evaluation of the cells equivalents or gene copies present in a sample include TaqMan chemistry. While each of the two measures can tell you different things about your

assay, our approach for these assays was to use gene copies. It has now become routine to produce double stranded DNA of sequences of 125 base pairs or longer with high fidelity. These products are commercially sold and are called GBlocks. In this project, GBlocks for the 127 and 68 base pair bovine and porcine sequences, respectively, were purchased. For the 68 base pair porcine sequence, a random sequence of twenty-five C, G, A, and Ts were added to each of the 5' and 3' ends of the sequence to accomplish a 125 base pair strand of DNA. These GBlocks were quantified, serially diluted, and used to evaluate assay performance and build standard curves. The standard deviation between replicates indicated the precision of the assay as well as the micropipetting skill of the analyst. The efficiency of the standard curve (E) indicated the performance of the primer and probe concentrations/sets at the concentrations used. To assess the recoveries of the extraction and clean-up portions of this method, a known quantity of GBlock DNA was spiked into sample PEG pellets (matrix spikes) and carried through the assay. The extent of agreement of the final quantity of gene copies were evaluated in order to determine assay recovery efficiency and the extent of PCR inhibition. The level of detection (LOD) and level of quantification (LOQ) can be determined for this assay by testing aliquots of GBlocks that have been diluted to levels calculated to contain single and fractions of gene copies.

Validation of Methods Using Environmental Samples

The final value of these assays is whether they can be used with environmental samples with high agreement of true positives and negatives with infrequent occurrence of false positives and negatives. In addition, infrequent occurrence of high levels of PCR inhibition is also desirable. In order to assess whether the proposed assays meet these requirements, two separate strategies were employed. To assess the occurrence of true and false positives and negatives, a field sample survey was used. A number of fecal samples, urine samples, and lagoon/slurry samples from cows, pigs, a variety of other animals and wastewaters were used to challenge each assay (BAdV, PAdV and HAdV).

As part of the field survey, a total of twenty-two fecal samples were collected at the Wisconsin State Fair in August 2016. Sampled animals included cows, pigs, horses, ducks, goats and chickens. Samples were used to test the performance of the qPCR assays and assess the presence of adenoviruses in individual cows and pigs. The fecal samples were processed using the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA), and the DNA extracts were stored at -80°C until use.

Additionally, samples from eight different farms across the state have been collected. Fecal samples were extracted and analyzed on a wet weight basis. Urine and lagoon/slurry samples were concentrated with PEG precipitation and extracted with the Zymo kit.

As part of the field survey, the assays were also used to analyze sewage samples (settled sewage and effluent). Samples from five different Publicly Owned Treatment Works (POTWs) were collected in the winter of 2016 and again in spring 2017. Samples were concentrated with PEG precipitation and extracted with the Zymo kit. The results of all of the samples included in the field sample survey are presented in the results section below.

To assess the performance of the assays with environmental samples, once the assays have been evaluated through steps 1 through 5 above, the assays were used to analyze on-going Large Volume RTCR Assessment samples. The results of these assays were be compared to the conclusions provided by integrating the sanitary survey, indicator organism enumerations, API 20E identifications, and existing MST assay results (*Bacteroides* sp., *Bifidobacteria* sp., *Rhodococcus coprophilus,* and human Adenovirus). The project team partners with WDNR staff across the state to collect, concentrate and analyze these RTCR samples.

Results

Bovine Assay

In the design of the bovine AdV, this project uses the 127 base pair target sequence of the bovine AdV, as well as the forward and reverse primers researched by Pedersen (Pedersen *et al.,* 2011). In Pedersen's research, a SYBR green assay was used as the detection system. SYBR green binds to double stranded DNA and can lead to false-positive results in the presence of environmental DNA that can anneal at temperatures above or at the annealing temperature used in the thermal program, 58°C here. Thus, the use of a probe designed using TaqMan® chemistry can be hypothesized to increase the specificity and reproducibility of the assay across various water matrices. This project designed a probe for the bovine AdV with the ThermoFisher TaqMan® specifications in order to potentially decrease false-positive results. These specifications are (1) the melting temperatures of primers should be 58-60˚C and the probe 10 ˚C higher, (2) oligomers should be 15-30 bases in length, (3) oligomers should have 30-80% G+C

content, (4) in the last five nucleotides at the 3' end the oligomer should have no more than 2 G+Cs, (5) the ideal length of amplicon is 50-150 bases, (6) the oligomer should contain more C's than G's and no G at the 5' end. In addition, the probe should not have a potential to hair pin, where it binds to itself, and not self-anneal for a successful assay. Using these parameters, four probe designs were developed with the OligoCalc utility created by Kibbe, 2007. No single probe design met all of the above criteria. Therefore, these designs were then compared as shown below in Table 2, and assessed for which one contained the least significant deviation from ideal.

Table 2. Designs for Bovine AdV Probe

Design	Bases (5' to 3')	Melting Temp, ˚C	% of C+Gs	C vs. G	Hair pin	Self-Anneal
1	A ACGAGTTGCG GCTGATCCAA T	54.8	50%	5 C, 6 G	no	yes
2	**ACGAGTTGCG GCTGATCCAA TTTATCA**	**60.1**	**46%**	**6 C, 7 G**	**no**	**no**
3	ACTTAATG GTGCTAAATG CAGAGGGTCAAC	60.3	43%	5 C, 8 G	no	no
4	CAGAGGGTCA ACGAGTTGCG GCTGATCCA	65.7	58%	7 C, 10 G	yes	no

*G and Cs in the last five 3' nucleotides are colored red
Chosen design is bolded in table

The probe chosen for initial experimental evaluation in this project was design 2. This design was chosen over design 1 and 4 because it did not self-anneal or hair pin. This design was selected over design 3 because although it does not contain more cytosines than guanines, it is closer to equal Cs to Gs and it contained a higher percentage of C and Gs. The thermocycling program chosen was based on the one tested by Pedersen *et al.* (2011). Thus, summarized in Table 3, is the bovine adenovirus assay evaluated in all bovine experiments conducted this project period. The HEX dye was used for the probe because its peak emission wavelength does not overlap with that of the porcine dye, FAM. This is important for testing the potential to multi-plex the assays which cannot be conducted if dye pairs emit at overlapping wavelengths, it will artificially lower the Ct values.

Table 3. qPCR Summary of Bovine AdV

Target	Primers and Probes (3' to 5')	Thermocycling Conditions
Bovine Adenovirus (BAdV10) (127 base pairs)	Forward B10F: TTACGCCCAACTTCCTTTTG Reverse B10R: CCACGCGTCTACTCCGTATT 127 Probe: BoAdV10: HEX-ACGAGTTGCGCCTGATCCAATTTATCA-1ABkFQ	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 58°C 40 sec, 72° C 1 sec; 60° C 60 sec

Testing Efficiency of Bovine Assay

The first experiment to test efficiency of the designed bovine probe was varying the probe concentrations. Typically, concentrations between 200 and 600 nM are used in TaqMan® qPCR assays. However, in certain virus assays, probe concentrations as high as 900 nM were required for optimal detection (Plummer and Long, 2013). So initially, the probe concentrations tested were from 200 to 600 nM. The final assay probe concentration should have the highest efficiency (E between 90 and 105 percent) and most linear standard curve (highest R^2s value). An experiment was conducted spiking Type I lab water with known quantities of target oligomers (G-Blocks) in triplicate, the results are summarized in Table 4. G-Blocks are known concentrations of synthetic sequences of DNA that match the target sequence.

Table 4. Bovine Probe Concentration Experiment

	200 nM	300 nM	400 nM	500 nM	600 nM
Efficiency %	91.60	80.74	85.53	89.87	90.26
Linear Fit Equation (y = Ct; x = gc)	y = -3.5411x + 41.875	y= -3.8902x + 42.599	y= -3.7257x + 43.13	y= -3.5798x + 42.517	y= -3.5914x + 42.718
R^2	0.9961	0.993	0.9906	0.9871	0.9966

Results with this particular probe sequence appeared to be highly successful. Experiments to determine the most effective probe concentration were conducted. Further experimentation with additional probe sequences did not appear warranted. This probe was included in the final recommended assay (see Appendix).

When comparing the efficiency, probe concentrations of 200 and 600 nM were the only two above 90%, with 200 nM demonstrating the highest efficiency. Therefore, probe concentrations of 200 and 600 nM were concluded to yield the highest amplification efficiencies. When comparing the R^2 values of the $\log_{10}$ linear equation for the standard curves amplified, probe concentrations of 200 and 600 nM were above 0.996, with the experiment using 600 nM yielding the most linear standard curve. This may be an artifact of pipetting efficiency, and further investigation was warranted. The following experiments were conducted and tested using these two most promising probe concentrations. The goal was to assess the most effective probe concentration to include in the final assay. The first experiment analyzed "synthetic samples" of Type I lab water processed using polyethylene glycol precipitation (PEG; SOP in Appendix to this report) and AdV bovine G-blocks. The lab water was prepared with first concentrating the sample DNA using polyethylene glycol (PEG) precipitation. Then the DNA was extracted using the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA; SOP shown in the Appendix to this report). The samples were spiked at different steps of preparation, in duplicate, to determine the loss of DNA at each step. The sample was spiked before the virus concentration (PEG), after the concentration but before the DNA extraction (pellet), and after extraction (extract). Three replicate reactions of each sample produced were assayed using qPCR. The mean results for each treatment in this first experiment are shown below in Figure 1.

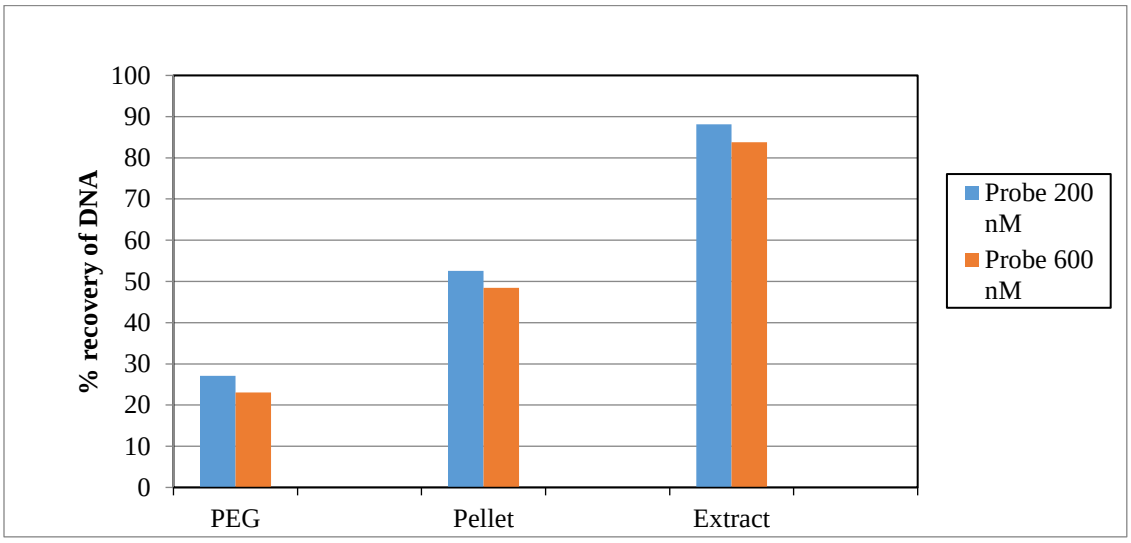


Figure 1. Performance of Bovine Probe Concentrations with Spiked Lab Water

The results demonstrate that the 200 nM probe concentration yielded the highest recovery of gene copies in all three cases. Recovery of free DNA, G-Block, was affected by each processing

step. Approximately 25% recovery was observed for spikes directly into Type I lab water, 50% was recovered when pellets were spiked and processed using MoBio PowerSoil DNA Isolation kit, and over 80% of anticipated gene copies were detected when the nucleic acid extract was spiked. At this juncture, it was asked whether the choice of clean-up kit, selected for the ssDNA Torque Teno Virus (TTV) was also the most appropriate for dsDNA Adenovirus. Thus, further experiments comparing different clean-up kits were planned, results are presented below.

The next experiment tested the 200 nM and 600 nM probe concentrations with Lake Mendota water. The lake water experiment tested the performance of the probe concentrations, as well as the detection of the target in the presence of natural environmental inhibitors (ions and organic matter) and competition (natural microbial community). The results from this first lake water experiment are summarized below in Figure 2. The samples were processed as above using the PEG concentration step and MoBio PowerSoil DNA extraction kit. Again duplicates of each spike level were prepared and triplicate qPCR reactions of each duplicate were assayed,

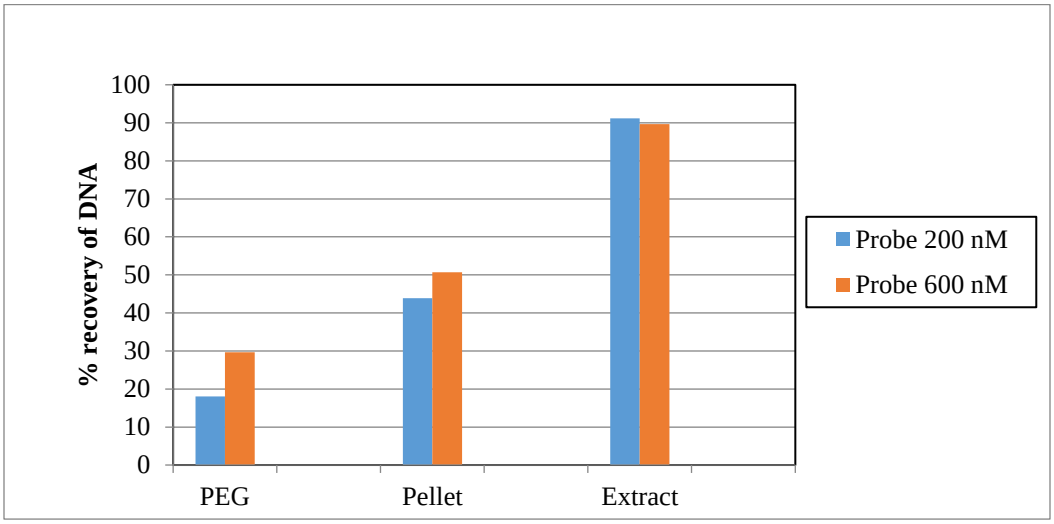


Figure 2. Performance of Bovine Probe Concentrations with Spiked Lake Water

Using lake water as the matrix with MoBio PowerSoil DNA Isolation kit for extraction and clean-up, the experiment produced contradicting results. The highest DNA recovery for the PEG and pellet spikes was with the 600 nM probe concentration in the Lake Water experiment. While the highest recovery for the extract was with the 200 nM probe concentration. These results are hypothesized to result from either pipetting variation and/or the buffer used in the clean-up steps interacting with target DNA enabling higher detection with the 600 nM probe concentration.

A challenge when analyzing environmental samples using qPCR is the presence of substances that can inhibit the reaction. This lake water experiment, when compared to the Type I lab water without inhibitors, produced similar free DNA recoveries accounting for analyst variation. Overall, competing organisms and presence of natural PCR inhibitors do not appear to interfere significantly with the assay outcome when evaluating the total workflow of the assay. However, the loss of DNA signal from sample to extract appears important. This is not unlike other virus concentration assays (Shafer, 2000) where overall approximately 20 percent recovery was documented. This has significant impact on assessing the meaning of final results of "below detection limits" or "negative" results for an individual MST samples.

Comparison of Clean-up Kits

To further assess the appropriate probe concentrations, Lake Mendota water was again tested; however the sample DNA was extracted and cleaned-up using the Zymo DNA Isolation kit (Irvine, CA; SOP shown in the Appendix to this report). This experiment evaluated probe concentrations, the presence of competing microorganisms, the presence of inhibitors and extraction kit performance. The experiment concentrated the sample with PEG, but did not spike the sample at the PEG step to limit the number of samples and controls to those that could be analyzed on a single 96-well plate. Thus, in Figure 3 below, the only spikes were made to the pellets and extracts. As discussed above, extraction kits were compared to assess whether alternative methods would improve DNA recovery. In previous research conducted in the Long Lab, up to five different extraction and clean-up techniques were compared. Balancing complexity of method, labor required, DNA quantity and quality yield, the Zymo DNA Isolations Kit was selected for other microorganisms, such as *E.coli* (Mansour, 2014). Therefore, comparison of the MoBio and Zymo kits were compared. Duplicate spike samples were prepared, and triplicate qPCR reactions of each spike were assayed. The Figure 4 below shows the two Lake Mendota experiments, the one using the MoBio extraction kit and the other using the Zymo kit, side-by-side.

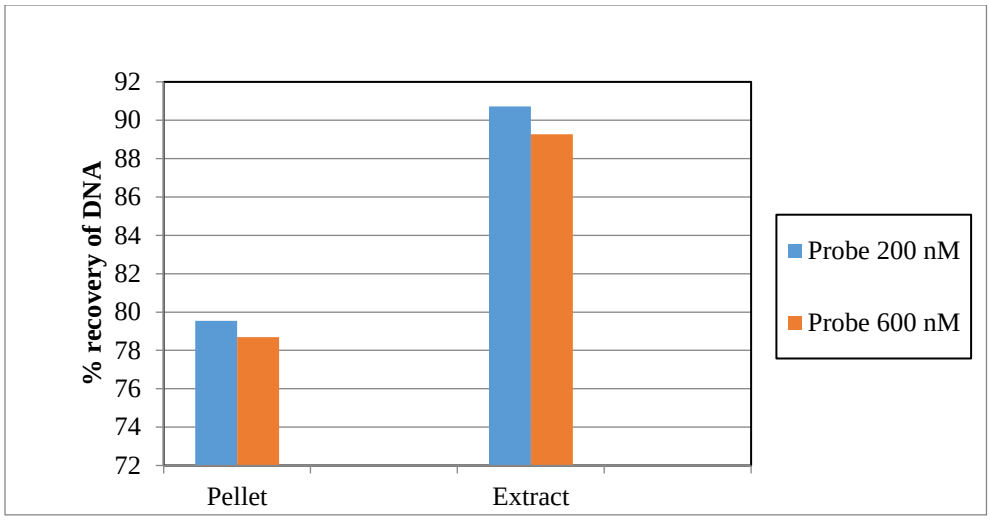


Figure 3. Bovine Probe Concentrations in Lake Mendota Water with Zymo Extraction

This experiment's results concluded the most effective probe concentration to be the 200 nM, although the differences may not be statistically significant. The 200 nM concentration yielded the highest DNA gene copy recovery for each step. Although the 600 nM probe was more effective with the MioBio extraction kit, from the results in Figure 4 below, the 200 nM probe was more effective with the Zymo DNA Isolation extraction kit.

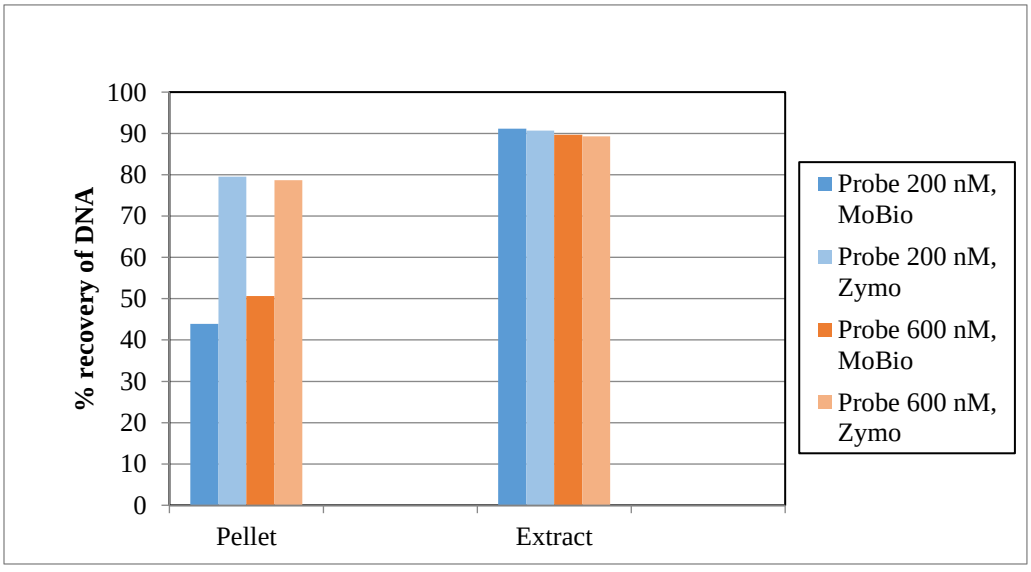


Figure 4. Bovine Probe Concentrations in Lake Mendota Water Comparing Extraction Kits

In comparing the data from the two extraction/clean-up methods, it can be concluded that the Zymo DNA Isolation kit extraction resulted in the highest recovery. The results show that the highest amount of G-Block DNA was detected with the Zymo extraction kit for the spiked pellet

step. For the spiked pellet samples, the Zymo recovered on average 79% which was almost double that of the average recovery using the MoBio kit, 47%. When spiking the final nucleic acid extract, the MoBio kit recoveries were similar. Considering the importance of erroneous conclusions from a "below detection limits" or "negative" sample, it is recommended that the final protocol transferred to the fee-for-service laboratory setting include the Zymo kit, see *Recommendations* below.

Quantification – Gblocks

The last experiment in the development of the bovine assay evaluated competition with the porcine AdV DNA target. This experiment used Lake Mendota water as the matrix with the 200 nM probe and the Zymo DNA Isolation extraction kit. The pellets and extracts were spiked with 100 times more G-Block porcine AdV than bovine AdV G-block. additional samples were spiked with 100 times more bovine than porcine and equal amounts of each G-block. The results of the experiment, in Figure 5, present the average of two replicates analyzed.

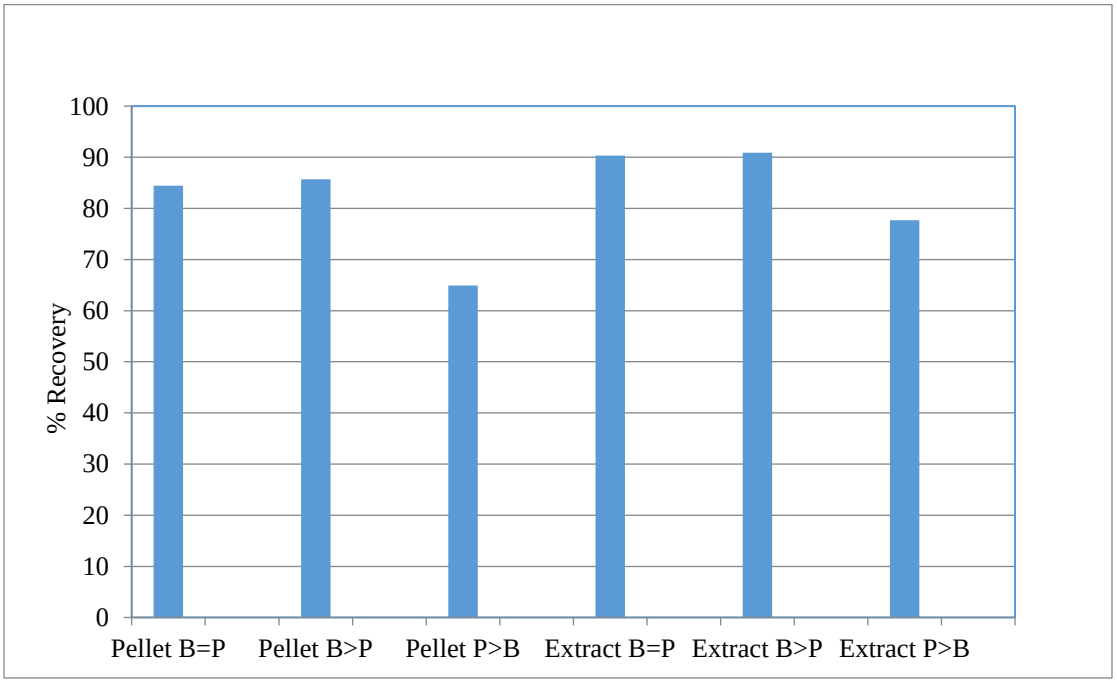


Figure 5. Bovine Target Competition in Lake Mendota Water with Zymo Extraction

From the results, when the porcine virus is 100 times more concentrated than the bovine, the assay detects 20% less bovine AdV DNA in the spiked pellet samples than when the bovine and

porcine are equal. The assay also detects 13% less bovine AdV DNA in the spiked extract samples. This demonstrates that the bovine assay is affected when there are large amounts of competing DNA. However, when the competition of high amounts of organisms, either specifically porcine or in lake water, the assay still performs well as seen by the high DNA recovery percentages, over 60 percent in all instances.

Multi-plex Analysis

Owing to logistical limitations, one experiment was conducted to compare single-plex and multi-plex analyses using the program summarized in Table 5, below. The results are summarized in Table 6, below. When comparing results from the single-plex and multi-plex assays for bovine adenovirus the advantage is the ability to analyze for both bovine and porcine targets in the same qPCR run. The samples in the multi-plex cycled quicker to yield Ct values than the samples that were tested using the bovine only assay. This creates efficiency by saving time and labor evaluating two targets in the same PCR run. There was some variation in exact gene copies quantified with the singe-plex assay appearing to detect higher numbers than the multi-plex format. The fecal sample that cross-reacted and tested positive for porcine adenovirus in the multi-plex was orders of magnitude lower than the bovine target. Unfortunately, there was not enough extract to analyze for the porcine target in single-plex format. It is recommended that additional testing should be carried out to determine if this is consistent for samples spiked with known amounts of either bovine or porcine GBlocks and samples spiked with known amounts of both GBlocks. The potential for time and labor savings could outweigh potential losses in sensitivity (loss of detection of a proportion of gene copies).

Table 5. Summary of qPCR Conditions for Single-plex and Multi-plex Assays

Target	**Primers and Probes (5' to 3')**	**Thermocycling Conditions**
Bovine Adenovirus (BAdV10) (127 base pairs)	Forward B10F: TTACGCCCAACTTCCTTTTG; 300 nM Reverse B10R: CCACGCGTCTACTCCGTATT ; 300 nM Probe BoAdV10 P451: HEX-ACGAGTTGC/ZEN/GGCTGATCCAATTTATCA-IABkFQ; 200 nM	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 58°C 40 sec, 72° C 1 sec; 60° C 60 sec
Porcine Adenovirus (PAdV) (68 base pairs)	Forward Q-PAdV-F: AACGGCCGCTACTGCAAG; 900 nM Reverse Q-PAdV-R: GCAGCAGGCTCTTGAGG; 900 nM Probe Q-PAdV-P: 6FAM- CCACATCCAG/ZEN/GTGCCGC-IABkFQ; 225 nM	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 55°C 20 sec; 60° 20 sec
Multi-plex BAdV10 and PAdV	Same as above	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 55°C 20 sec; 72° C 1 sec; 60° 45 sec

Table 6. Results of Analysis with Single-plex and Multi-plex qPCR for Bovine Target

			Single-plex		Multi-plex			
Sample	Kit	# Tested	Bovine		Porcine		Bovine	
			# positive	gc/ 100mL	# positive	gc/ 100mL	# positive	gc/ 100mL
Lagoon Slurry (cow)	Zymo	10	10	$5.7x10^3$ - $1.1x10^9$	1	22	10	$2.1x10^2$ - $1.9x10^9$
Urine		2	2	$1.2x10^2$ - $4.6x10^2$	0	BDL	2	$1.2x10^4$ - $4.7x10^4$
			#positive	gc/ g wet weight	#positive	gc/ g wet weight	#positive	gc/ g wet weight
Feces	MoBio	10	0	BDL	0	BDL	1	$9.4x10^4$

Porcine Assay

The porcine AdV uses the 68 base pair target sequence of the porcine AdV, researched by Hundesa (Hundesa *et al.*, 2009). This assay design is shown below in Table 7. Hundesa *et al.* optimized the assay using the Stratagene thermocycler with 0.225 µM of the probe and 10µL of template DNA in a 30 µL reaction of Universal MasterMix (Applied Biosystems, Carlsbad, CA). Most qPCR assays conducted at WSLH use 5 µL of template and Environmental Master Mix (Applied Biosystems, Carlsbad, CA) in a 30 µL reaction. It has been demonstrated that assay performance can be significantly affected by thermocycler platform. Therefore, the design of the porcine assay was focused on optimizing the template sample DNA concentration for use in WSLH on the ABI 7500 platform. Much of the testing performed was conducted in parallel to those conducted for the bovine AdV assay described above.

Table 7. qPCR Summary of Porcine AdV

Target	**Primers and Probes**	**Thermocycling Conditions**
Porcine Adenovirus (PAdV) (68 base pairs)	Forward Q-PAdV-F: AACGGCCGCTACTGCAAG Reverse Q-PAdV-R: GCAGCAGGCTCTTGAGG Probe Q-PAdV-P: FAM-CACATCCAGGTGCCGC-BHQ1	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 55°C 20 sec; 60° 20 sec

Testing Efficiency of Porcine Assay

The first experiment to test efficiency of the modified porcine assay varied the sample template DNA volumes. The sample template DNA volumes, or the extracted nucleic acids, are important because different concentrations can produce different efficiencies as they bring along with them competing DNA and residual inhibitors. The experiment tested three sample DNA volumes in triplicate reactions: 5, 7.5 and 10 µL with positive porcine GBlock spikes. The first experiment, results shown in Figure 6, was conducted with Type I lab water and the same spiking steps as the bovine experiments above. This experiment used the MoBio PowerSoil extraction kit.

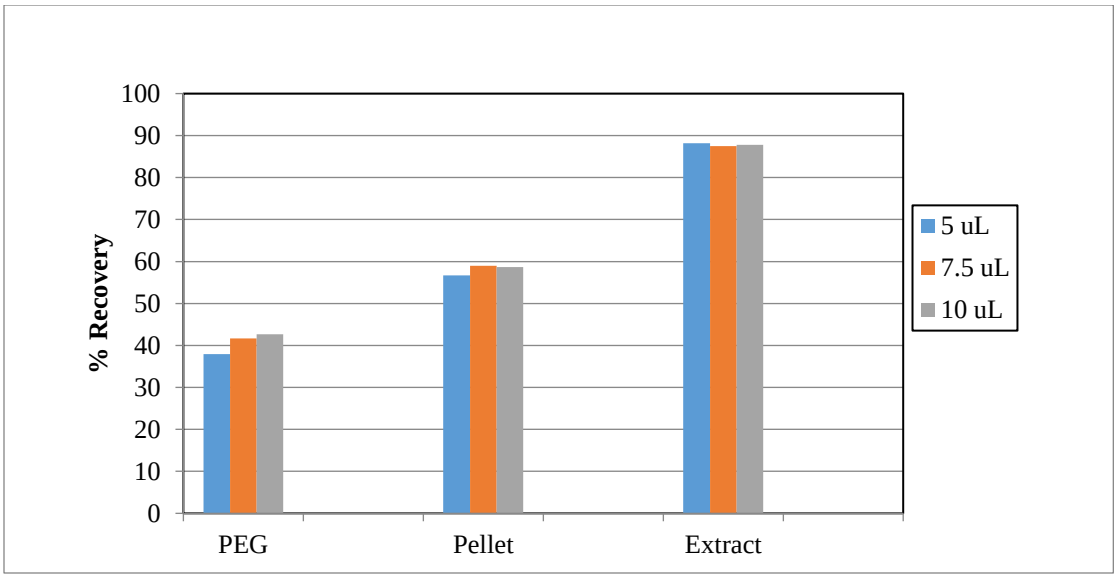


Figure 6. Porcine Template DNA Concentrations on Lab Water with MoBio Extraction

The results of this experiment show DNA recoveries are very similar in each step regardless of template volume used. Variations in specific recovery efficiencies among all the spikes tested were observed; however, those differences are not statistically significant. Overall, the sample volume assayed does not appear to make a large difference in this assay design when the matrix has been subject to kit clean-up. Further experimentation varying template DNA volumes using Lake Mendota as a matrix is discussed below.

The second experiment again varied the template DNA volume, but this time the matrix was Lake Mendota water. This tested the various DNA volumes in the presence of competing microorganisms and natural environmental inhibitors. The MoBio PowerSoil extraction kit was used. The results are summarized in Figure 7.

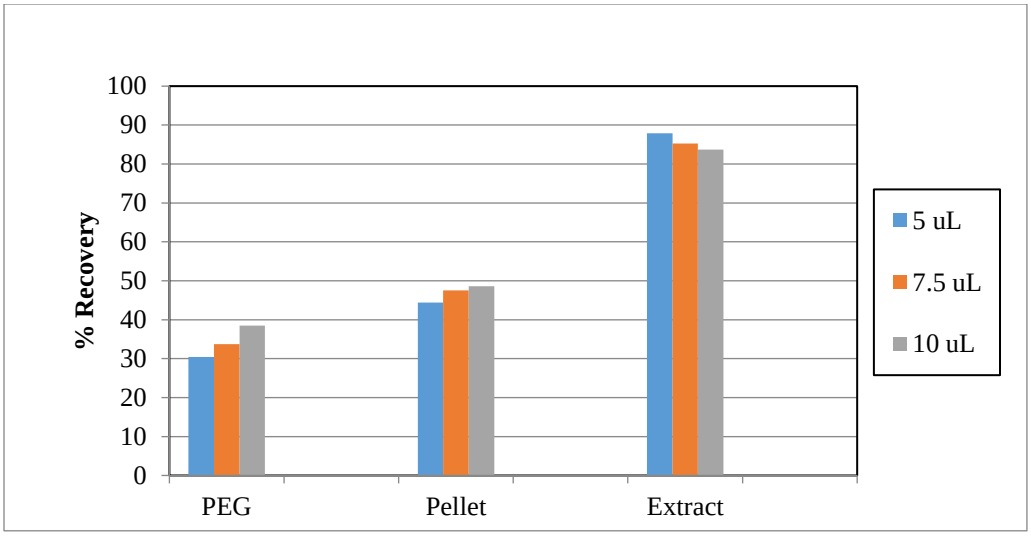


Figure 7. Porcine Template DNA Experiment on Lake Mendota water with MoBio Extraction

The results from this experiment show that again all template volumes yielded very similar recovery efficiencies. The variation among spiked samples and template volumes are larger on average than those observed in Type I lab water, a very clean matrix. Again, the variation between volumes was not statistically significant. Observationally, the 5 µL volume test for spiked extract appeared more efficient than the other template volumes although the opposite was observed for the spiked pellet and PEG liquid. These results were normalized to the actual gene copies spiked into the sample and the theoretical gene copies calculated to be present in the volume tested. It is clear that target volume has the potential to affect assay results. Although, the variation in results enumerating targets present in low concentrations in environmental samples may mask this level of variation.

Nucleic Acid Extraction and Purification

The next experiment once again compared two DNA extraction kits also using three template DNA volumes. This experiment sampled Lake Mendota water and extracted the G-Block DNA with the Zymo DNA Isolation extraction kit. As in the bovine experiments, duplicate spikes (pellet and extract) were prepared, and each spike was assayed using triplicate qPCR reactions. The experiment, results in Figure 8, tested competing organisms, inhibitors and extraction kits.

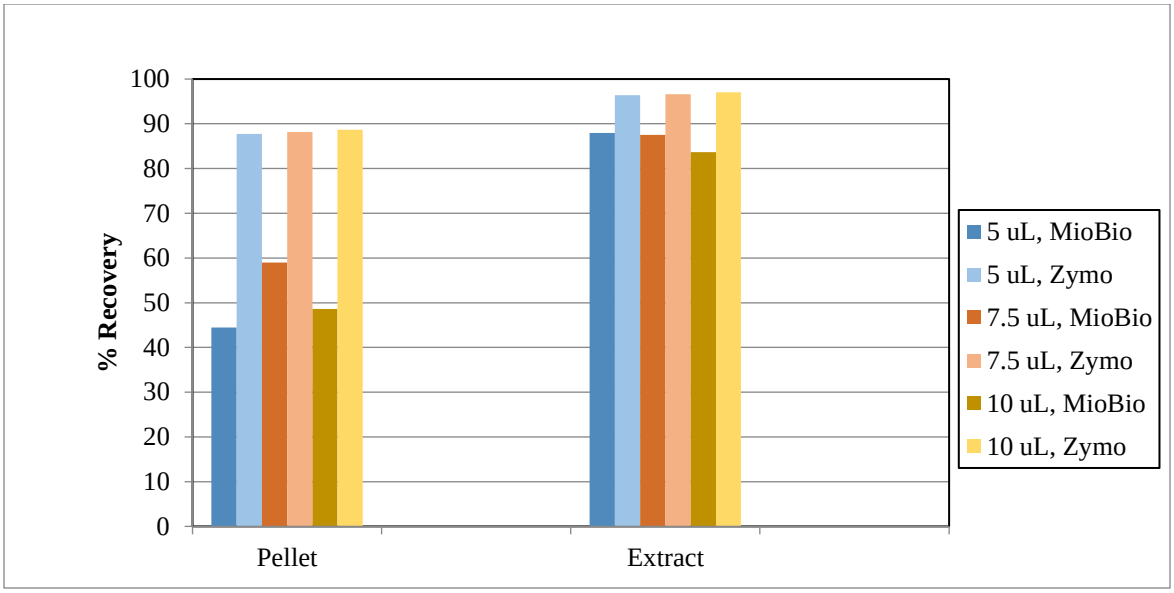


Figure 8. Porcine DNA Experiment on Lake Mendota Water with Extraction Kits

For all sample DNA volumes and both spiking steps, the Zymo extraction kit outperformed the MoBio extraction kit. It detected about 20% more porcine AdV target gene copies for each template volume with the spiked pellets and about 10% more AdV DNA with the spiked extracts. Therefore, along with the bovine extraction kit results (Figure 4), the Zymo extraction kit was chosen as the final extraction kit to be implemented for these source tracking targets. Curiously, it appears that variation in recovery and detection efficiency among various volumes of target used in the PCR reaction was not a factor when the Zymo extraction kit was used.

The last experiment challenging the performance of the porcine assay was the competition with bovine AdV DNA. This experiment used the Zymo extraction kit on Lake Mendota water and 5 µL sample DNA. Again, duplicate spiked pellets and extracts with 100 times more bovine AdV G-Block than porcine AdV G-Block, 100 times more porcine than bovine, and the equal amounts of each AdV G-Block were tested. Each spike was assayed in triplicate using qPCR. The results of the experiment, in Figure 9, show the average of two replicates of the two different spikes that were analyzed.

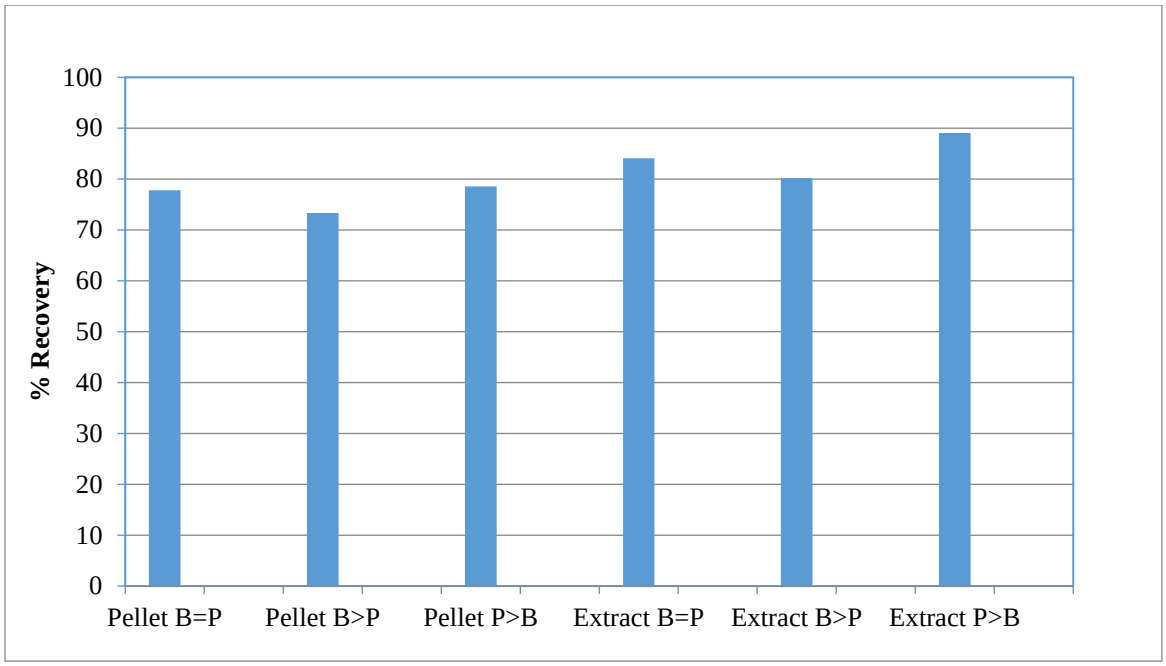


Figure 9. Porcine Virus Competition in Lake Mendota Water with Zymo Extraction

From the results, when the gene copies of bovine AdV is 100 times more prevalent than the porcine AdV, the recovery of the porcine target was 5% less in the spiked pellet samples than when the bovine and porcine gene copies were equal. The assay also detected 9% less in the spiked extract samples when the bovine gene copies were 100 times more prevalent. This shows that the porcine assay will be less affected when there are large amounts of other types of DNA than the bovine assay. This may be a result of the characteristic of the bovine probe not meeting all the criteria for an ideal TaqMan® probe, see discussion above. The porcine assay detects close to or over 80 percent of spiked porcine gene copies when there are large amounts of competition. When the competitions of high amounts of organisms, either specifically bovine AdV DNA or in lake water, the assay still performs well and further experimentation with additional challenges to the assay does not appear warranted.

Quantification – Gblocks

Similar to experiments for the bovine assay, recovery of porcine AdV free DNA, G-Block, was affected by each processing step. Approximately 40% recovery was observed for spikes directly into Type I lab water, 60% was recovered when pellets were spiked and processed using MoBio PowerSoil DNA Isolation kit, and 90% of anticipated gene copies were detected when the

nucleic acid extract was spiked. As discussed for the bovine adenovirus assay the overall workflow recovery efficiency has implications for interpreting "below detection limit" or "negative" MST samples.

Validation of Methods Using Environmental Samples

As discussed above, a field survey suite of animal fecal samples, animal urine samples, farm lagoon/slurry samples and municipal sewage samples were analyzed using the bovine and porcine adenovirus assays developed and tested, as discussed above. Because of numerous variation in sample collection and handling resulting from different staff, the results for these analyses are grouped and presented in appropriate sampling effort campaigns.

State Fair Manure Samples

Beginning this reporting period, animal fecal samples were tested with the designed assays. Initial specificity of the assay has been assessed by using the optimized assays to analyze a variety of individual animal fecal samples to test cross reactivity. Cross-reactivity has been demonstrated for a number of assays, in particular the *Bacteroides* spp. assays for human and bovine targets developed by Layton *et al.* (2006) and the human *Bifidobacteria* assay developed by Gomez-Donate *et al.* (2012). During the 2015 and 2016 Wisconsin State Fairs, a total of 43 animal fecal samples were collected and duplicate aliquots of approximately 0.10 to 0.25 g wet weight were frozen for molecular extraction. The DNA from these samples were extracted, cleaned up, and tested with each assay to determine whether there is cross reaction with feces of other animals. Pig and cow samples were collected as well as those from goats, sheep, horses, chickens, geese, ducks, alpacas and lambs. The samples have been collected from a broad geographical area across the state to prove insight into the applicability of the assays as a statewide MST tool. The samples from 2015 and 2016 were collected from farms statewide in the counties with the blue star shown in Figure 10. The DNA from the approximately 0.25 g wet weight of fecal samples of the 2015 and 2016 State Fair were extracted with the MoBio PowerSoil DNA Isolation kit. Since the Zymo ZR Soil Microbe DNA MiniPrep™ Extraction kit was newly chosen as the more efficient kit, an additional six cow manure samples were collected and extracted with the Zymo kit and tested in a comparison with the MoBio extracts. However, since the fecal samples from animals that are shedding adenoviruses should contain high titers of

virus, specific extraction kits should not be as critical in obtaining the data necessary to determine the strength of the assays to provide MST information.

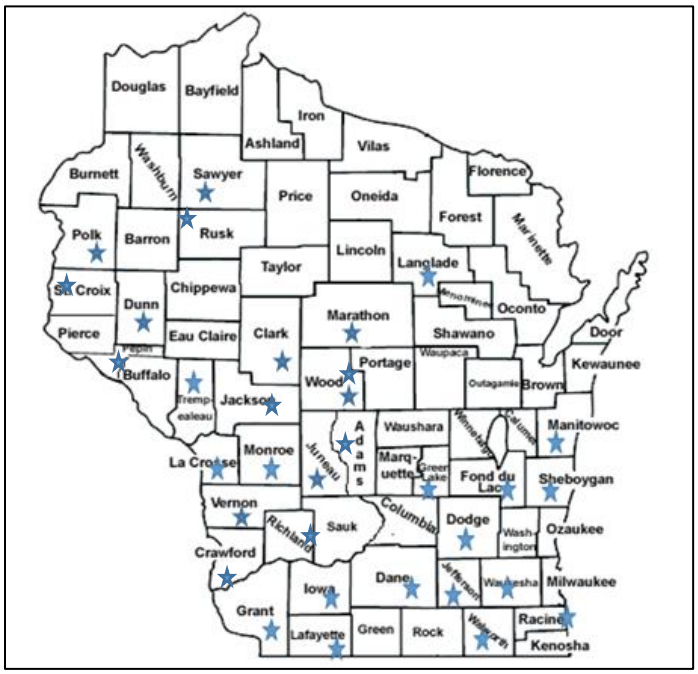


Figure 10. Fecal Samples Collected at the WI State Fair

The results from the fecal samples proved the specificity of each assay. The fecal sample extracts were tested using three replicates each by qPCR. The results for the bovine adenovirus target are summarized in Table 8 and for the porcine adenovirus target in Table 9. There were low levels of cross reaction among horse, goose, and chicken feces with the porcine assay.

Overall, 1 animal out of 58 (1.7%) of the Wisconsin State Fair samples tested positive for bovine adenovirus suggesting that either bovine adenovirus is not very prevalent or that it does not shed in high enough quantities to be detected in feces (Table X). The sheep that tested positive for bovine adenovirus yielded a gene copy number that suggests the bovine adenovirus may potentially infect other animals in the Bovidae family, specifically the Ovis genus.

Table 8. Bovine Adenovirus qPCR 2015 State Fair Results

Sample	Extract Kit	# tested	# positive	gc/ g wet weight
Pig	MoBio	6	BDL	BDL
Cow		15	BDL	BDL
Horse		5	BDL	BDL
Chicken		8	BDL	BDL
Lamb		3	BDL	BDL
Goose		7	BDL	BDL
Goat		5	BDL	BDL
Sheep		3	1	$2.7x10^2$ **
Alpaca		1	BDL	BDL
Duck		1	BDL	BDL
				gc/ 100mL
Cow urine	Zymo	4	BDL	BDL

BDL - below detection limit
** denotes an estimation of 0.25g was used for the wet weight sample tested

Overall, 14% of animal samples from the Wisconsin State Fair tested positive for porcine adenovirus (Table Y). Fifty percent of pig fecal samples tested positive for porcine adenovirus while cross-reaction among all other animal samples were between 12.5-20% positive. The gene copies of porcine adenovirus present in horse samples was on the same order of magnitude as the pigs, suggesting the virus could be potentially infecting equine species. The other farm animals that tested positive for porcine adenovirus (cow, chicken, and goose) showed gene copy numbers that were a full order of magnitude lower than porcine and equine feces suggesting they may become inoculated and shed the porcine adenovirus without it infecting their systems.

Porcine adenovirus was present in one of the horse samples at the same order of magnitude as the swine fecal samples. The animals were in separate barns and the samples collected with single use, sterile scoops, so it is therefore more likely that equine species have the ability to

propagate the porcine adenovirus. Further testing should be conducted to determine what the likelihood of horses co-raised on farms with swine carry or propagate the virus. Also, a comprehensive analysis of all livestock for cross-reactivity with porcine adenovirus will inform and influence water safety concerns as well as mitigation strategies for well-head protection and surface water pollution. This future research is critical to fully evaluate the use of the porcine assay for source tracking.

Table 9. Porcine Adenovirus qPCR Results for State Fair Fecal Samples

Sample	Extract Kit	# tested	# positive	gc/ g wet weight
Pig	MoBio	6	3	$1.9x10^2$ - $3.4x10^3$ **
Cow		16	2	$1.5x10^2$ - $2.2x10^2$ **
Horse		5	1	$3.9x10^3$ - $5.1x10^3$ **
Chicken		8	1	$3.6x10^2$ - $5.4x10^2$ **
Lamb		3	BDL	BDL
Goose		7	1	$1.9*10^3$
Goat		3	BDL	BDL
Sheep		3	BDL	BDL
Alpaca		1	BDL	BDL
Duck		1	BDL	BDL
				gc/ 100mL
Cow urine	Zymo	4	BDL	BDL

BDL - below detection limit
****** denotes an estimation of 0.25g was used for the wet weight tested

Municipal Sewage Samples

To assure that the sequences selected for each of the adenovirus assays are not present in viruses that cross infect humans, wastewater sewage samples were collected and tested. A series of municipal sewage samples were collected from five Publicly Owned Treatment Works (POTWs) from Brown, Dane, Milwaukee, Ozaukee, and Walworth counties were concentrated with

polyethylene glycol (PEG) precipitation, extracted with the Zymo ZR Soil Microbe DNA MiniPrep™ Extraction kit and tested with the bovine, porcine, and human AdV assays. A total of five diverse sewage district sites were sampled. The treatment plants are indicated by average daily flows. Samples were collected and tested during the winter months as some of the plants do not institute disinfection between November and April. The disinfection would significantly lower the virus count of the effluent sample. Two split samples of the settled sewage and two split samples of the final effluent were analyzed from each site. The assays' tested three replicates of each PEG extract using qPCR.

The results of the human AdV assay are summarized in Table 10. These analyses used the human AdV assay developed by Jothikumar *et al.* (2005) and modified by Plummer and Long (2013). From the results, human AdV was prevalent in all settled raw sewage samples on the order of 10^8 gene copies per 100 mL. The human AdV was present in four of the plant's final effluent samples on the order of 10^5 gc/100 mL. After discussion with the 3 MGD plant, it was determined that disinfection was in use. Therefore, it can be concluded that the disinfection process destroyed the AdV genetic material. Lower numbers to levels below detection limits of human AdV in the 100+MGD effluent sample was likely a result of their use of UV disinfection.

Table 10. Analysis for Human Adenovirus in Municipal Sewage

Location	Sample	Replicate 1 (gc/100 mL)	Replicate 2 (gc/100 mL)
42 MGD plant	Effluent	8.66×10^4	9.18×10^4
	Settled Sewage	1.13×10^6	1.59×10^6
100+ MGD plant	Effluent	4.70×10^3 *	BDL
	Settled Sewage	1.72×10^6	3.547×10^5
30 MGD plant	Effluent	9.91×10^4	8.80×10^4
	Settled Sewage	1.25×10^6	1.60×10^6
3.9 MGD plant	Effluent	9.43×10^4	8.81×10^4
	Settled Sewage	1.10×10^6	3.56×10^9
3 MGD plant	Effluent	BDL	BDL
	Settled Sewage	1.63×10^{10}	5.75×10^4

*only one of three replicate volumes of this sample was positive upon PCR analyses

All of the settled sewage samples were positive for human adenovirus although the orders of magnitude varied greatly. This variation in results indicate that the reproducibility between samples collected and split on the same day may be highly variable. It was already observed in the development of the assays, that levels of 40 percent or more of target can be lost in various steps of sample concentration, extraction and analyses. Again, this has significant implications for the interpretation of "below detection limit" or "negative" MST samples.

The same sewage extracts were tested for bovine and porcine adenovirus targets. The bovine assay showed no cross-reactivity, as there were no positives for bovine assay, and a positive for the porcine target was detected in the 3 MGD plant effluent (198 gc/100 mL compared to the human target of 10^4 to 10^{10} gc/100 mL). It is thought that this could be a result of tanker delivery of restaurant waste into the feed at that plant. This stream contains meat washings and would explain the very small numbers for this detection.

The same treatment plants were sampled in the spring of 2017, but analyzed for only the bovine and porcine targets. All samples remained negative for the bovine target. The difference between winter and spring samples for the porcine target demonstrated one low level positive to no positives.

Evaluation of storage of extracts was assessed by analyzing the winter municipal sewage samples twice for the human adenovirus target on February 20, 2017 and again on March 3, 2017 as a result of some quality control sample failures. All sample DNA signal had decayed between 0.1 to 13 percent between analyses, with an average loss of 2.9 percent of signal based on resulting Ct values. These data indicate that it is important to include a "calibration" standard consisting of nuclease free water spiked with a known quantity of GBlock and carry it through with the samples if exact quantification is important. The decay in signal in the calibration standard can be used to account for loss of DNA during sample storage.

Farm Samples

In order to provide a slightly more robust field sample analysis, a number of samples including feces, urine, and lagoon/slurry samples were collected at cow and swine farms. Approximately 0.25 g of feces were extracted using the MoBio or Zymo kit, depending on the analyst. Urine and lagoon/slurry samples were PEG precipitated and extracted. The results are summarized in Table 11 below.

Table 11. Porcine and Bovine AdV qPCR Results for Fall 2017 Farm Sampling

Sample	Extract Kit	# of tested	Porcine AdV		Bovine AdV	
			# positive	gc/ g dry wt	# positive	gc/ g dry wt
Pig feces	MoBio	9	4	$5.9x10^2$ - $2.2x10^5$ **	0	BDL
Cow feces		2	0	BDL	0	BDL
			# positive	gc/ 100mL	# positive	gc/ 100mL
Pig urine	Zymo	3	2	$2.9x10^3$ - $3.0x10^4$ **	0	BDL
Lagoon slurry (pig)		2	1	$3.2x10^5$ - $4.5x10^5$ **	0	BDL

BDL- below detection limit
****** denotes an estimation of 0.25g was used for the wet weight

All samples were negative for the bovine adenovirus target. This is not surprising, as prior results demonstrated that bovine adenovirus is more typically recovered from cow urine than cow feces (Pedersen *et al*., 2011). Overall, 4 of 9 pig fecal samples from these farms tested positive for porcine adenovirus while 2 of 3 pig urine samples and 1 of 2 pig lagoon slurries tested positive. This indicates that liquid samples may give a more accurate depiction of the adenovirus concentration that farms may be adding to their surrounding watersheds. The total gene copies present in feces, urine, and lagoon slurries were on the same order of magnitude suggesting that there is no preferential biological pathway for how the porcine adenovirus is shed by the animal.

Well Investigation Samples

Among 74 transient non-community water systems experiencing total coliform rule positive samples, eleven samples tested positive for other MST targets in the State Laboratory of Hygiene toolbox. Four samples negative for all MST targets were selected from similar geological settings and system characteristics as the positive samples. One enforcement sample, with a suspected manure contamination was analyzed during the project period. All samples were tested for the presence of bovine and porcine targets. The results are summarized in Table 12. The

majority of samples contained evidence of human-associated microorganisms. The majority of these samples did not test positive for the bovine or porcine targets. The presence of *R. coprophilus*, the grazing animal indicator, did not correlate with detection of the bovine target (LE and RTO). However, the sample that was grossly contaminated with manure (Enforcement) tested positive for both bovine and porcine targets. At the LAN site, based on sanitary survey data, indicated that the site was served by an on-site septic system but was also adjacent to a field where recent manure spreading was evident.

Table 12. Well Investigation Sample Results

Sample ID	MST Testing	Bovine (gc/100 mL)	Porcine (gc/100 mL)
MG	Human *Bacteroides*	BDL	BDL
AUYA	Human *Bacteroides*	BDL	BDL
AYA2	Negative	BDL	BDL
LE	Human *Bacteroides* & *R. coprophilus*	BDL	BDL
LE2	Negative	BDL	BDL
CHEM	Human *Bacteroides*	BDL	BDL
DMS	Human *Bacteroides*	BDL	BDL
CVL	Human *Bacteroides* & Human Adenovirus	BDL	BDL
SW	Human *Bacteroides*	BDL	BDL
HL2	Human Adenovirus	BDL	BDL
RTO	*R. coprophilus*	BDL	BDL
BSC	Human *Bacteroides*	BDL	BDL
LAN	Human *Bacteroides*	$1.3x10^4$	$1.0x10^4$
Hill	Negative	BDL	BDL
UMC	Negative	BDL	BDL
Enforcement	*R. coprophilus*	4	6

Overall, these results support the caveat for all MST testing, that negative samples are difficult to interpret. The assay may not have a low enough detection limit for sites where significant attenuation of the target occurs during transport between the source and sampling site. The presence of both targets in the two samples with evidence of contamination by other assays, indicate the difficulty of interpreting assay data as stand alone values. Never the less, testing of source samples (feces, urine and wastewater) does indicate that the bovine and porcine adenovirus assays may provide additional information when used in a toolbox with other MST assays and sanitary survey and land use information.

Recommendations and Conclusions

Overall, the suite of assays developed for bovine, porcine, and human adenovirus targets using qPCR demonstrate significant promise in applications for source tracking at the Wisconsin State Laboratory of Hygiene and elsewhere. The bovine and human targets do not appear to yield non-specific detection, based on fecal and farm samples tested. An interesting finding is the presence of the bovine target consistently in urine and barn washings (slurries and lagoons) buy not in individual fecal samples. The porcine target is infrequently detected in non-swine samples such as sewage and fecal samples of other livestock. It is therefore recommended that all positive results be evaluated in coordination with land use and sanitary survey information. Negative results must be evaluated with extreme caution. For example, bovine manure spreading from piles that contain only manure, and not urine, could be negative for bovine adenoviruses and thus a manure contaminated water sample would also be negative. Contamination that is distant in time and geography from a manure source could test negative as a result of attenuation and inactivation of adenoviruses over time and space.

Figure 11 and Table 13 summarize the potential work flow and polymerase chain reaction programs for using bovine, porcine and human adenoviruses as microbial source tracking tools, respectively. Detailed "standard operating procedures" for each step in the process are contained in the Appendix to this report.

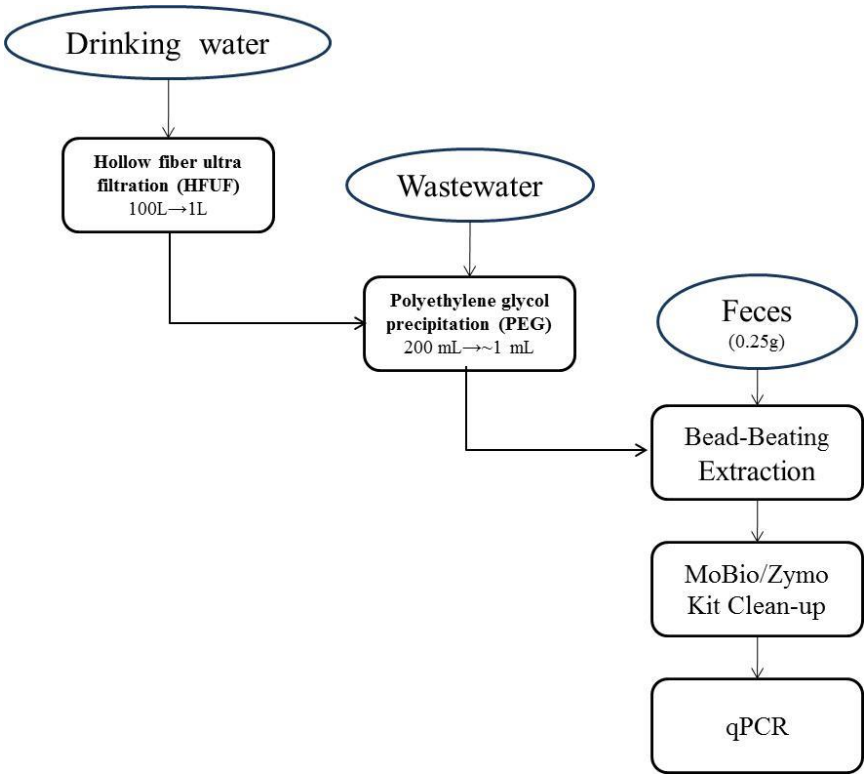


Figure 11. Sample Process Flow for Adenovirus Source Tracking

Table 13. Adenovirus qPCR Assays

Target	Primers and Probes (5' to 3')	Thermocycling Conditions
Bovine Adenovirus (BAdV10) (127 base pairs)	Forward B10F: TTACGCCCAACTTCCTTTTG; 300 nM Reverse B10R: CCACGCGTCTACTCCGTATT ; 300 nM Probe BoAdV10 P451: HEX-ACGAGTTGC/ZEN/GGCTGATCCAATTTATCA-IABkFQ; 200 nM	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 58°C 40 sec, 72° C 1 sec; 60° C 60 sec
Porcine Adenovirus (PAdV) (68 base pairs)	Forward Q-PAdV-F: AACGGCCGCTACTGCAAG; 900 nM Reverse Q-PAdV-R: GCAGCAGGCTCTTGAGG; 900 nM Probe Q-PAdV-P: 6FAM-CCACATCCAG/ZEN/GTGCCGC-IABkFQ; 225 nM	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 55°C 20 sec; 60° 20 sec
Multi-plex BAdV10 and PAdV	Same as above	95°C 10 minutes Cycle (n=45): 95°C 15 sec; 55°C 20 sec; 72° C 1 sec; 60° 45 sec
Human Adenovirus (73 base pairs)	Forward JTVXF: GGA CGC CTC GGA GTA CCT GAG Modified Reverse mod-JTVXR: ACI GTG GGG TTT CTR AAC TTG TT Probe JTVXP: CTG GTG CAG TTC GCC CGT GCC A	95°C 10 minutes Cycle (n=45): 95°C 10 sec; 55°C 30 sec; 72° C 15 sec

References

Bofill-Mas, S., Pina, S., & Girones, R. 2000. Documenting the epidemiologic patterns of polyomaviruses in human populations by studying their presence in urban sewage. *Applied and Environment Microbiology*, **66**(1): 238–245.

Gòmez-Donaté, M., E. Ballesté, M. Nuniesa, and A.R. Blanch. 2012. New molecular quantitative PCR assay for detection of host-specific *Bifidobacteriaceae* for microbial source tracking. *Applied and Environmental Microbiology*, **78**(16): 5788-5795.

Green, H.C., R.A. Haugland, M. Varma, H.T. Millen, M.A. Borchardt, K.G. Field, W.A. Walters, R. Knight, M. Sivaganesan, C.A. Kelty, and O.C. Shanks. 2014. Improved HF183

quantitative real-time PCR assay for characterization of human fecal pollution in ambient surface water samples. *Applied and Environmental Microbiology*, **80**(10): 3086-3094.

Hundesa, A., C. Maluquer de Motes, S. Bofill-Mas, N. Albinana-Gimenez, and R. Girones. 2006. Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Applied and Environmental Microbiology*, **72**: 7886–7893.

Hundesa, A., C. Maluquer de Motes, N. Albinana-Gimenez, J. Rodriguez-Manzano, S. Bofill-Mas, E. Sunen, and R. Girones.2009. Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment. *Journal of Virological Methods*, **158**: 130-135.

Jothikumar, N. et al. "Quantitative Real-Time PCR Assays For Detection of Human Adenoviruses And Identification Of Serotypes 40 And 41". *Applied and Environmental Microbiology* **71.6** (2005): 3131-3136.

Kibbe, W.A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Res*. **35**: W43-W46.

Kildare, B.J., Leutenegger, C.M., McSwain, B.S., Bambic, D.G., Rajal, V.B., Wuertz, S., 2007. 16S r RNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: a Bayesian approach. *Water Research*, **41**(16): 3701–3715.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R., and Sayler, G. 2006. Development of *Bacteroides* 16S rRNA Gene TaqMan-Based Real-Time PCR Assays for Estimation of Total, Human, and Bovine Fecal Pollution in Water. *Applied and Environmental Microbiology*. **ò**(6): 4214-4224.

Lipp, Erin K., Samuel A. Farrah, and Joan B. Rose. 2001. Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community. *Marine Pollution Bulletin*, **42**(4): 286-293.

Long, S.C., C. Arango P., and J.D. Plummer. 2005. An Optimized Enumeration Method for Sorbitol-Fermenting *Bifidobacteria* in Environmental Samples. *Canadian Journal of Microbiology* 51(5): 413-422.

Long, S.C., J. Olstadt, and B.D. Moss. 2015. Fecal source tracking and water quality at WSLH. Presentation to the Wisconsin Department of Natural Resources, June 16 (2015), Madison, WI.

Mansour, I.M. 2014. Optimization and Assessment of Performance of a qPCR Method for the Detection of Shiga Toxin-Producing *Escherichia coli* in Environmental Waters Optimization and Assessment of Performance of a qPCR Method for the Detection of Shiga Toxin-Producing *Escherichia coli* in Environmental Waters. Master's Thesis, Soil Science, University of Wisconsin, Madison, WI.

Mara, D. D. and Oragui, J. I. 1981. Occurrence of *Rhodococcus coprophilus* and associated actinomycetes in feces, sewage, and freshwater. *Applied and Environmental Microbiology* **42**(6), 1037-1042.

Mara, D. D., and Oragui, J. I. 1983. Sorbitol fermenting *Bifidobacteria* as specific indicators of human fecal pollution. *Journal of Applied Bacteriology*, **55**(2), 349-358.

McQuaig, S. M., Scott, T. M., Lukasik, J. O., Paul, J. H., & Harwood, V. J. 2009. Quantification of human polyomaviruses JC Virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. *Applied and Environment Microbiology*, **75**(11), 3379–3388.

Merrett-Jones, M., Morris, R., Coope, R., & Wheeler, D. 1991. The relationship between enteric viruses and indicators of sewage pollution in UK seawaters. In Proceedings of the UK Symposium on Health-related Water Microbiology (pp. 158-164). IAWPRC London.

Pedersen, J.A., K.D. McMahon, S.C. Long, and S.D. Sibley. 2011. Fecal source tracking using human adenovirus and polyomaviruses. WRI Project Number WR09R002 Report. Madison, WI.

Plummer, J.D., and S.C. Long. 2009. Identifying Sources of Surface Water Pollution: A toolbox approach. *Journal of the American Water Works Association*, **101**(9): 75-88.

Plummer, J.D., and S.C. Long. 2013. Assessment of Torque Teno Virus as a viral pathogen indicator in waters. Water Research Foundation, Denver, CO.

Polaczyk, A. L., Narayanan, J., Cromeans, T. L., Hahn, D., Roberts, J. M., Amburgey, J. E., and Hill, V. R. 2008. Ultrafiltration-based techniques for rapid and simultaneous concentration of multiple microbe classes from 100-L tap water samples. *Journal of Microbiological Methods*, **73**(2): 92-99.

Rådström, P., Knutsson, R., Wolffs, P., Lövenklev, M., and Löfström, C. 2004. Pre-PCR processing, strategies to generate PCR-compatible samples. *Molecular Biotechnology*, **26**(2): 133-146.

Rowbotham, T.J., and T. Cross. 1977. Ecology of *Rhodococcus coprophilus* and Associated Actinomycetes in Fresh Water and Agricultural Habitats. *Journal of General Microbiology* **100**(JUN): 231-240.

Savill, M.G., S.R. Murray, P. Scholes, E.W. Maas, R.E. McCormick, E.B. Moore, and B.J. Gilpin. 2001. Application of Polymerase Chain Reaction (PCR) and TaqMan PCR Techniques to the Detection and Identification of *Rhodococcus coprophilus* in Faecal Samples. *Journal of Microbiological Methods* **47**(3): 355-368.

Shanks, O.C., E. Atikovic, A.D. Blackwood, J. Lu, R.T. Noble, J. Santo Domingo, S. Seifring, M. Sivaganesan, and R.A. Haugland. 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. *Applied and Environmental Microbiology*, **74**(3): 5507-5513.

Shanks, O.C., C.A. Kelty, M. Sivaganesan, M. Varma, and R.A. Haugland. 2009. Quantitative PCR for genetic markers of human fecal pollution. *Applied and Environmental Microbiology*, **75**(17): 745-752.

Sibley, S. D. 2009. Personal Communication. Department of Soil Science, University of Wisconsin, Madison, WI.

Shafer, E.J. 2000. The Suitability Of F+ RNA Coliphages as Source Identifiers of Fecal Pollution in the Wachusett Reservoir Watershed. Master's thesis, Civil and Environmental Engineering, University of Massachusetts, Amherst, MA.

Sobsey, M.D. and O.D. Simmons. 2009. Final Report: Simultaneous Concentration and Real-time Detection of Multiple Classes of Microbial Pathogens from Drinking Water. Environmental Protection Agency, Office of Drinking Water, Washington, DC.

Appendix

Standard Operating Procedures

- Hollow fiber ultrafiltration (HFUF)
- Polyethylene glycol precipitation (PEG)
- Zymo Kit extraction
- MoBio Kit extraction
- Bovine Adenovirus MasterMix Template
- Porcine Adenovirus MasterMix Template
- Human Adenovirus MasterMix Template
- Multi-plex bovine and porcine adenovirus MasterMix Template

Dead-End Hollow Fiber Ultrafiltration for Field Filtering Public Water Supply Well Samples

March 9, 2016, rev. January 5, 2017

The purpose of this procedure is to concentrate large volumes (approximately 100 L) of well water in order to conduct in depth assessment of the source(s) of coliforms/RTCR unsafes. This method has been tested for efficacy with bacteria (*E. coli* and enterococci), viruses (coliphage, adenovirus, norovirus), and parasites (aerobic endospores as a surrogate, *Cryptosporidium*, and *Giardia*). This method is a modification of the method validated by WSLH for preparedness response incorporating modifications per Smith and Hill (2009).

Media and Reagents

5% newborn calf serum (or fetal bovine serum)
114mL sterile cell culture water
6mL calf serum
Prepare day of filter blocking
Filters may be blocked the evening prior to sample filtration if kept refrigerated
(this is enough for 1 filter)

1000X NaPP solution
*Sent pre-made from WSLH or county health department
10 g sodium polyphosphate
100 mL sterile cell culture water
In sterile container, heat in 65ºC waterbath to dissolve (may need to be warmed overnight)
Store at room temperature for up to 3 months

Filter Pre-Wash Solution
1 L sterile Type I laboratory water
1 mL 1000X NaPP solution
Prepare day of use
(this is enough for 1 filter)

Filter Post-Wash Solution
900 mL sterile Type 1 laboratory water
0.09 mL TWEEN® 80
0.9 mL 1000X NaPP solution
9.0 µL Antifoam Y-30
Prepare day of use
(this is enough for 1 filter)

10% Sodium thiosulfate
100 g sodium thiosulfate
1,000 mL sterile cell culture water
Autoclave, 15 min, 121ºC
Store at room temperature

(for chlorinated samples only)

Apparatus and Materials (in order of assembly)

Portable HFUF kit

Bag of 6 mL tubes of frozen calf serum (completely non-toxic and non-hazardous), remove and thaw **only** the number of tubes needed

20 L or 10 L carboys – sterilized (Fisher 02-960-20B)

Spare (empty) 1 L bottle for sample collection – sterilized

Backwash collection bottles – 1L, empty, pre-weighed, sterilized

2 filtrate (waste) buckets or carboys (or wastewater can be discharged to the sewer or onto the ground in appropriate circumstances)

Funnel – cleaned with bleach water, rinsed three times with tap or sample water, and covered with aluminum foil prior to use

ThermoSafe cooler for shipping samples

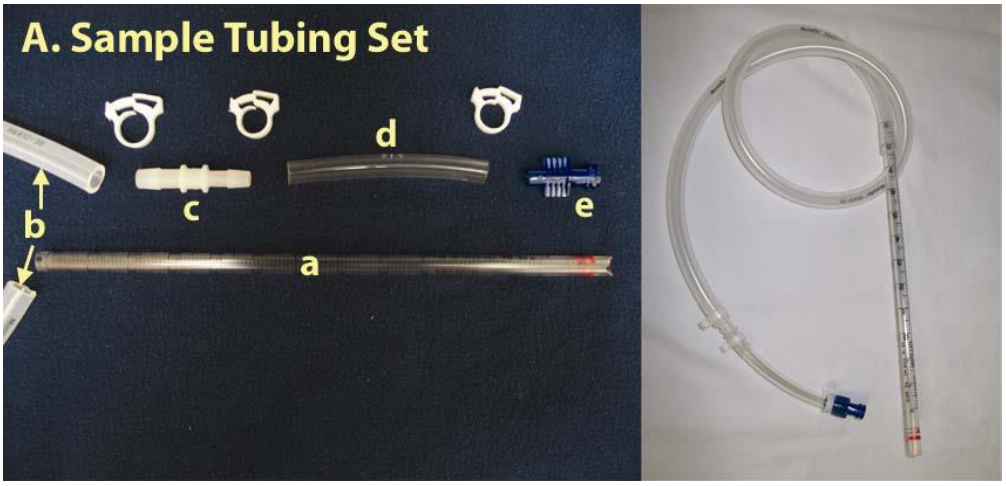
<u>Items you will find in your portable HFUF kit*</u>

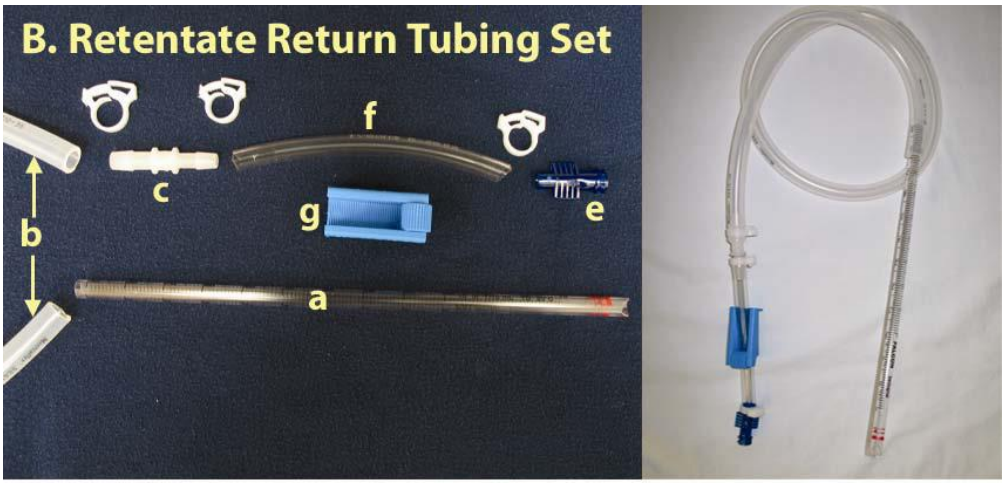
- 3 rectangular plastic bins
- Bags of various sized gloves
- Bag of antiseptic wipes
- Bag of Wypall L40 wipes (absorbent laboratory diapers)
- Bag of trash bags and zip-top bags
- Sharpie marker
- Bag of ATP sample bottles
- Bag of coliform sample bottles with Styrofoam packer
- 3 Asahi REXEED 21S filters
- 3 sterile containers of 114 mL of cell culture water
- Bag of 60 cc syringes
- Bag of 20 mL or 10 mL tubes of 1000X NaPP solution
- Bag of 10 mL or 5 mL tubes of 10% sodium thiosulfate solution
- Bottle holder containing
    - 3 bottles for preparing pre-wash
    - 3 bottles for preparing post-wash (containing 100 µL of TWEEN 80)
- Bag of 1 mL tubes of 1000X NaPP solution
- Bag of 0.9 mL tubes of 1000X NaPP solution
- Bag of Antifoam Y-30 tubes (contains only µLs, do not be alarmed if tube looks empty)
- Bag of sterile transfer pipettes
- 3 Sample tubing sets (check expiration date)
- 3 Retentate tubing sets (check expiration date)
- 3 Filtrate tubing sets (check expiration date)
- 3 Backwash tubing sets – 33 inches (check expiration date)
- 25-foot, 1” diameter tube in a closable, cylindrical, plastic bucket
- Bag of zip ties
- Metal peg board hook

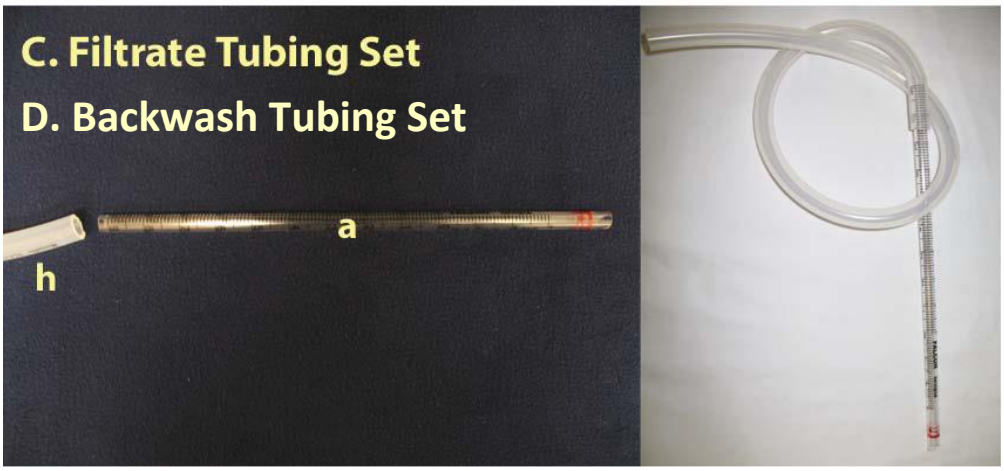
- MasterFlex L/S Easy-Load II Pump Head (Fisher 77201-62) mounted on MasterFlex L/S Precision Drive (Fisher 0752810)
- MasterFlex power cord
- Extension cord
- Lighter
- Pipette bulb
- 50 mL pipettes
- Filtrate tubing clamp
- Zip tie cutters
- Zip top bag containing paperwork (sanitary survey, HFUF protocol, HFUF bench sheet, sample request form, supply re-stock sheet, shipping cooler checklist)
- Bag of large aluminum foil sheets
- Bag of small aluminum foil sheets

*because of space considerations, some items may be packed in your ThermoSafe box

Recognizing Your Tubing Sets







Constructing Your System

I. Preparations

1. Open field kit and remove the inverted gray bin from right-hand side.
2. Remove pump and foam padding from field kit. Remove these 3 pieces individually to prevent damage to the pump.
3. Lift out only one (1) of the gray bins on the right-hand side containing water bottles, box of filtration accessories, etc. Leave one bin to collect spilled water.
4. Flip the previously removed empty gray bin upside-down so the open side is facing downwards. Place the right lip of this bin beneath the left lip of the gray bin currently in the field kit. Slide bin into place until it is flush with the bottom of the field kit.
5. Place the pump (without foam padding) and the 6 1-L bottle holder on the left-hand bin as shown in the photo below.
6. Record all data, or attach stickers (if applicable), on the bench sheet provided in the zip top bag labeled “Paperwork”.
7. Put on gloves. To maintain best aseptic practices, wipe gloves with antiseptic wipes.
8. Pretreat/block one dialysis filter per sample to be filtered (up to 3) with 5% calf serum solution (can be prepared in the office/lab the evening prior to use as long as it is kept refrigerated).
    - 8.1. Thaw one tube of 6 mL frozen calf serum (per sample) at room temperature.
    - 8.2. Pour thawed calf serum from tube into bottle containing 114 mL of sterile cell culture water. Cap the bottle and swirl to mix. Discard tube.
    - 8.3. Lay out a clean Wypall L40 on a clean surface.
    - 8.4. Using the zip tie cutters provided, carefully remove Asahi REXEED 21S filter from packaging. Place on clean Wypall L40 and remove end caps from filter. Do not remove the side caps. Do not discard packaging or end caps.

8.5. Position the Asahi REXEED 21S filter vertically with the top (orange end) facing up. Using a 60cc syringe (individually wrapped "BD 60 ml Syringe"), draw air into the syringe and then align with the top port (orange end). Slowly expel the contents of the 60cc syringe into the Asahi REXEED 21S filter, collecting the drained saline solution in a 5-gallon waste bucket. Repeat until all saline solution has been expelled (typically 3 or 4 times).

8.6. Position the Asahi REXEED 21S filter horizontally. Using the same 60cc syringe from Step 8.5, fill with 5% calf serum solution. Be sure to either leave the bottom port (blue end) cap off completely or attach loosely.

8.7. Align the 60cc syringe with the port on the top (orange end) of the Asahi REXEED 21S filter. Slowly expel the contents of the 60cc syringe into the Asahi REXEED 21S filter. Repeat until all solution has been used. If you are careful to keep the syringe aseptic, it may be used to block as many filters as needed (up to 3). Discard the 60cc syringe after all filters are blocked or the syringe becomes contaminated.

8.8. Seal the Asahi REXEED 21S filter end ports with end caps. Discard the bottle. Invert the filter at least 25 times to fully coat the filter with calf serum solution.

8.9. Label the Asahi REXEED 21S filter and a clean 2-gallon zip-top bag to correspond to the ID of the sample to be collected.

8.10. Place the blocked and labeled Asahi REXEED 21S filter back into its original packaging and place the packaged filter into the labeled zip-top bag.

8.11. Store filters in refrigerator or cooler with ice until use.

9. Prepare filter **pre-wash** and **post-wash** solutions (if desired, may be prepared in office/lab **same day** of filtering, prior to traveling to field site).

9.1. Obtain 1 L sterile water bottle marked "**Pre-Wash**" (1 for each sample). Pour the contents of one tube of 1 mL 1000X NaPP solution (located in Tupperware container) into 1 L bottle and label "**Pre-Wash**/*Sample Name*" on the labels provided on the bottle base and cap.

9.2. Cap tightly and mix "**Pre-Wash**/Sample" bottle by inverting 25 times. Use filter **pre-wash** solution same day of preparation.

9.3. Obtain 900 mL sterile water bottle marked "**Post-Wash**" (1 for each sample). Pour the contents of one tube of 0.90 mL 1000X NaPP solution into bottle and label "**Post-Wash**/*Sample Name*" on the labels provided on the bottle base and cap.

9.4. Using a new, sterile transfer pipette (located in Tupperware container), add a small volume of **post-wash** solution to tube containing Antifoam Y-30 (located in Tupperware container). Do not be alarmed if the tube appears empty because of the extremely small volume of Antifoam Y-30. Pipette up and down a few times to mix, the solution will appear milky-colored. Use transfer pipette to transfer solution to **post-wash** bottle and pipette up and down to rinse transfer pipette. Discard transfer pipette.

9.5. Cap tightly and mix "**Post-Wash**/Sample" bottle by inverting 25 times. Use filter **post-wash** solution same day of preparation.

II. Sampling*

10. Collect and prepare sample. **Be sure to collect initial ATP sample, followed by coliform sample, prior to rinsing previously used 20 or 10 L carboys, 1 L sample transfer bottles, or funnel, and prior to collecting HFUF samples.**

    10.1. Change gloves. Wipe gloves with antiseptic wipes.

    10.2. Remove autoclave tape from carboys.

    10.3. If previously used during this sampling period, rinse the inside of each carboy and 1 L sample transfer bottle 3 times with well water to get rid of residual bleach from Steps 14.4 through 14.6 and coat carboy/bottle walls with the sample to be collected. Dump rinse water into waste bucket or sewer.

    10.4. Pour the contents of one tube of 1000X NaPP solution (large tubes located in Tupperware container) into each of the 20 L sample carboys or 10 mL 1000XNaPP to each 10 L carboy. Discard tubes in the trash bags provided.

    10.5. If the sample is **chlorinated**, add 10 mL of 10% Na thiosulfate to each 20 L carboy or 5 mL of 10% Na thiosulfate to each 10 L carboy.

    10.6. Collect sample into each carboy containing 1000X NaPP solution (and Na thiosulfate if chlorinated). Fill to the 20 or 10 L mark on the carboys, which ever you are using. If space limitations prevent direct sample collection in carboy, use spare (empty) 1 L bottle provided to fill carboys to fill mark.

    10.7. Place the first sample carboy into the empty gray bin on the right side of the kit. Position the carboy so the volume markings are facing towards you (facing away from the field kit lid).

    *If using 10 L transfer containers, add 10 mL tube of 1000X NaPP, and fill to the 10L mark.

III. Constructing the System

11. Construct complete filtering set-up.

    11.1. Place the blocked filter into the mounting, with the blue end down and the orange end up, and the filter side ports pointing to the right (away from the pump).

    11.2. Zip-tie the filter to the filter mount as shown in the photo above.

    11.3. Make sure cap is tight on the lower side port of the filter.
    Note: Briefly inspect all connections on tubing units as you perform steps 11.4, 11.5, and 11.7 to make sure tubing clamps are positioned properly and connections are tight to minimize chances of leaks.

    11.4. Remove cap from upper side filter port and attach filtrate tubing set (C). Remove the pipette end of the filtrate tubing set (C) from the zip-top bag and place it into the waste bucket

    11.5. Remove cap from bottom filter port (blue end) and twist luer lock connector of retentate tubing set (B) into bottom filter port (blue end). Leave the pipette end in clean zip-top bag until ready for use in step 13.

    11.6. Ensure valve is **CLOSED** on retentate tubing (B).

III. Prewash and sample concentration

12. Pre-Wash.

12.1. Loosen the cap of the 1-L bottle of filter **pre-wash** solution prepared above that corresponds with the sample to be filtered. Remove pipette end of sample tubing set (A) from zip-top bag, quickly flame sterilize using the lighter provided in your kit, and place into the **pre-wash** solution bottle that corresponds with the sample to be filtered. Take care not to melt the pipette during flame sterilization.

12.2. Carefully remove the pipet end of the retentate tubing set (B), flame and place into waste bucket.

12.3. To wash residual calf serum out of filter, fully OPEN the flow regulator on the retentate tubing set (B). Feed the sample tubing set (A) into the pump head and plug in the pump. Turn the pump on using the switch on the back of the pump. Make sure the blue light indicating flow direction on the pump drive face is illuminated next to the picture with the arrow pointing towards the filter. Start pump by pushing the blue button on the far right hand side of the pump drive face. Using the up and down arrows, adjust the pump speed to 200 to 250. Be sure discharge is collected in disposal bucket or pumped to sewer. Once **pre-wash** bottle is empty, turn off pump by pressing the blue button on the far right hand side of the pump drive face.

12.4. Aseptically re-cap the **pre-wash** bottle for later use. Re-CLOSE the flow regulator on the retentate tubing set (B) and place pipet end back in Ziploc bag. Now you are ready to filter your sample.

13. Sample Concentration

13.1. Place first carboy inside gray bin in kit.

13.2. Using lighter provided, quickly flame sterilize the pipette tip from the sample tubing set (A). Take care not to melt the pipette. Aseptically place the pipette end into the first carboy containing NaPP treated sample (which should already be located in your field kit from previous steps). Repeat this step for the retentate tubing set (B). Both the sample (A) and retentate tubing set (B) pipettes should now be in the sample carboy.

13.3. Place funnel into first carboy. Ensure blue flow regulator on retentate tubing set (B) is now CLOSED as tight as it will go.
Note: If funnel was recently cleaned, wipe funnel off with a clean Wypall-L40 to remove any excess bleach solution.

13.4. Turn on pump. Adjust pump speed of 380 to 450. If this pump speed causes cavitation or sample tubing begins to leak, reduce pump speed slowly until cavitation or leaking ceases (typically 380-425).
Note: Dead-end HFUF runs at a higher pressure, so watching for tubing leaks is crucial to not lose any sample.

13.5. Place a new sheet of aluminum foil over top of funnel while filtering.

13.6. Use funnel to transfer contents of the second carboy into the first sample carboy.
Note: If funnel was previously used during this sampling period, rinse 3 times with current sample well water prior to this step to get rid of residual bleach from steps

16.4 and 16.6. Collect this rinse water in waste bucket or discharge directly to sewer.

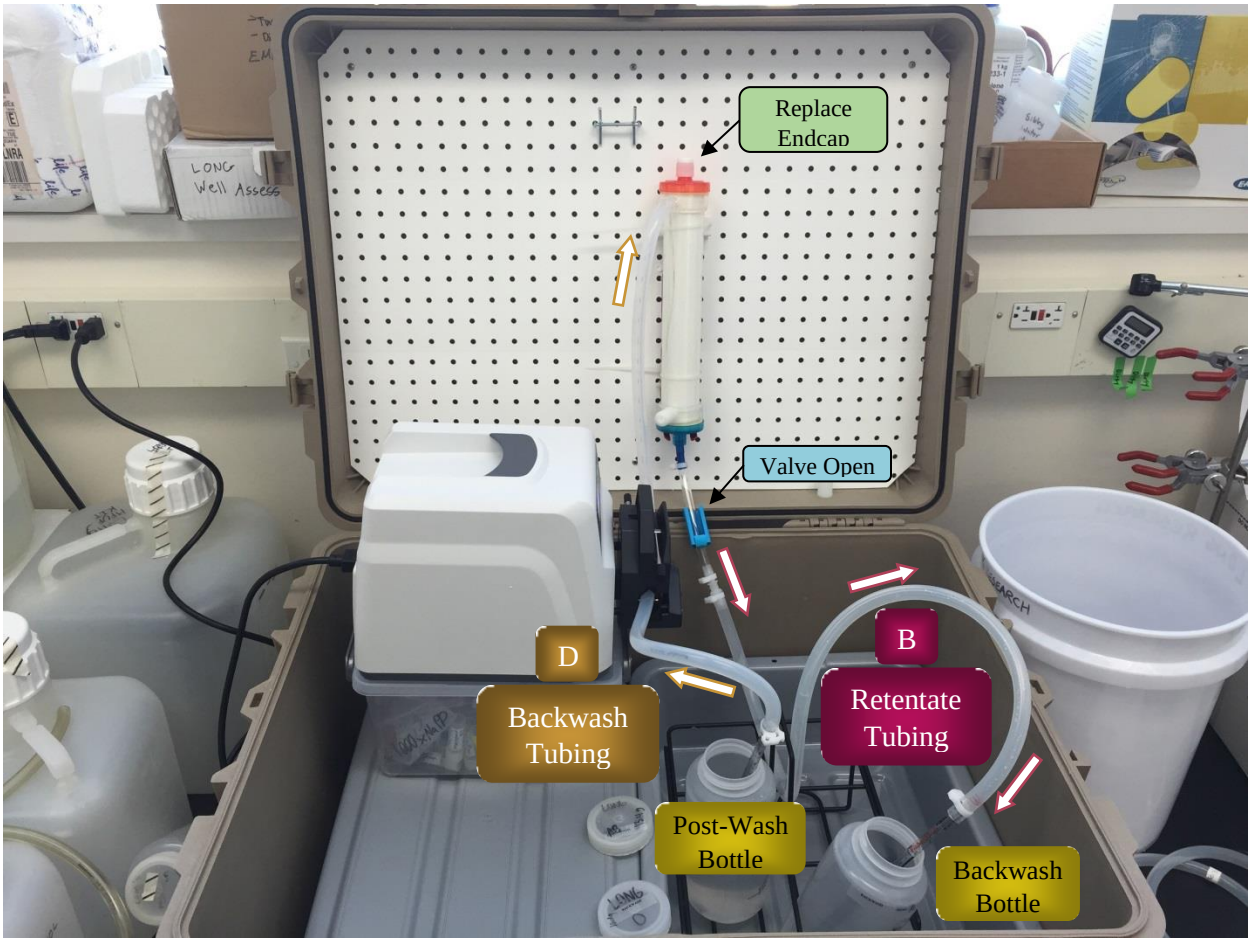
13.7. When waste bucket becomes full, switch to a second waste bucket while emptying the other.

13.8. Repeat steps 13.8 and 13.9 until all 100 L of volume has been transferred to the first carboy; continue filtering until volume in the first carboy reaches approximately 500-1000 mL. Turn off pump.

13.9. Swirl the remaining 500-1000mL around in the carboy to suspend and mix anything that may have settled during the concentration process. Transfer the remaining sample volume from the carboy into the empty **pre-wash** bottle (saved from above). **Be careful not to overfill the 1L pre-wash bottle if more than 1000mL is accidentally estimated**. Use a pipette bulb and 50 mL pipette tip to transfer remaining drops of sample from the carboy to the **pre-wash** bottle.

13.10. Turn on the pump and continue filtering all remaining sample (now in the **pre-wash** bottle). Turn off pump.

13.11. Release sample tubing (A) from the pump head. After inspecting the sample tubing (A) to make sure no spills will occur, detach the sample tubing (A) from filter and drain into **pre-wash** bottle. The volume in the pre-wash bottle should now be about 50-100 mL (a little more is not a problem as the amount remaining in the sample tubing may vary).
Note: When removing sample tubing from pump head, pressure built up from the concentration process may result in an "air burst" coming back from the filter through the tubing. Make sure to secure the tubing by holding the pipet end of the sample tubing in the **pre-wash bottle**.



14. The backwash process

    14.1. To backwash remaining particles from filter: remove filtrate tubing (C) and replace with new sterile backwash tubing set (D). Thread tubing through peristaltic pump. Flame pipet without melting it and place into **post-wash** bottle.

    14.2. Remove sample tubing (A), place it back in its zip top back for shipping to WSLH. Replace end cap onto top of filter.

    14.3. Place retentate tubing (B) into backwash bottle and fully open flow regulator.

    14.4. Ensure valve is **OPEN** on retentate tubing (B).

    14.5. With pump off, adjust pump speed to **200 rpm.**

    14.6. Turn on pump to pass **post-wash** through the filter and collect in backwash bottle. **Note:** Do not forget to reduce pump speed. If the pump is still set at concentration speed (400-450 rpm), the backwash tubing can be easily broken.

    14.7. Turn off pump. Collect the fluid remaining in the filter and tubing sets. **Note:** When removing backwash tubing from the pump head, it is possible another "air burst" may occur. If fluid is released back from the filter into the tubing, remember to save this volume in the backwash bottle. Also, a small volume of post-wash solution may remain in the post-wash bottle. This is okay; do not save this volume (it can be dumped out in a sink/drain).

14.8. After draining the tubing sets, place them back into their original bags for shipping to WSLH for cleaning and future reuse. Cap **backwash** bottle tightly, place in cooler.

V. Post-Filtration and Clean-up or Preparing for a Second Well

15. Package samples and equipment for shipment to WSLH.

15.1. Put all used ATP sample bottles and Colilert sample bottles into sample cooler.

15.2. Put all used **pre-wash** bottles, **post-wash** bottles, **backwash** bottles and bags of tubing sets into sample cooler.

15.3. Place sanitary survey(s), HFUF bench sheet(s), test request form(s), supply re-stock sheet, and shipping cooler checklist in a zip-top-bag and place bag in sample cooler.

16. Equipment clean-up/re-use for second sample.

16.1. Using the cutters provided, cut the zip-ties you used to attach filter to mounting. Discard zip-ties and filter. Change gloves. Wipe gloves and peg board with antiseptic wipes.

16.2. Be sure all items in portable HFUF kit and sample cooler are secured for transport.

16.3. Upon returning to your office or laboratory, rinse carboys, 1 L sample transfer bottle, and funnel with tap water and place upside-down on clean Wypall L40s to dry.

16.4. Prior to re-use, use funnel to fill each carboy with 10 L of tap water solution containing approximately 5% bleach (*i.e.* 9.5 L water to 500 mL household strength bleach). Remove funnel, rinse with tap water, dry with clean Wypall L40, and cover each opening with an appropriately sized aluminum foil sheet provided in your kit. Cap carboys tightly, shake, and dump. Rinse insides of carboys 3 times with tap water. Carboys and funnel are now ready for field deployment. Rinse bottle with water flushed from the well several times before collecting sample.

16.5. Prior to re-use, fill 1 L sample transfer bottle with 500 mL of tap water solution containing approximately 5% bleach (*i.e.* 475 mL water to 25 mL household strength bleach). Cap bottle tightly, shake, and dump. Rinse insides of bottle 3 times with tap water. The bottle is now ready for field deployment. Rinse bottle bottle with water flushed from the well several times before using to collect sample.

16.6. If collecting multiple samples on the same day, Steps 15.3 through 15.5 can be done in the field using well water from the next sample location **after the initial ATP and coliform samples have been collected from that well**. Proceed to Step 10.4.

REFERENCES

Smith, C.M., and V.R. Hill. 2009. Dead-End Hollow-Fiber Ultrafiltration for Recovery of Diverse Microbes from Water. *Applied and Environmental Microbiology* 75(16): 5284-5289.

Polyethylene Glycol Precipitation SOP

Water Samples

Version: January 27, 2011

Revised June 18, 2014; July 14, 2014; October 3, 2015; October 13, 2016

Materials

- Sterile graduated cylinders
- Sterile centrifuge tubes (choose appropriate size)
    - 50mL (make sure they are Corning rated for 15,500xg)
    - 250mL Corning
- Bacto Beef extract
- NaCl
- PEG 8000
- Alcohol burner
- Ethanol
- Absorbent diapers
- Sterile pipets
- Weigh boats
- Scoops
- Scale
- 5% Bleach solution
- Turn on Shaker Incubator set at 4°C, record that you will be using the incubator, what you are using it for, how long you will be using it, and at what temperature you have set it.
- 20 uL of 10^-4 G-block

Procedure

Day 1:

1. Measure 200mL of Lab water sample.
2. Aseptically pour sample into new, sterile 250mL centrifuge tubes containing 4.0 g beef extract (2% w/v final concentration), swirl to completely dissolve. **For 2 differen samples add 10 uL of 10^-4 G-block.**
3. Add 5µL anti-foam to underside of cap. Shake to mix.
4. Add PEG ingredients (**order is important!**):
    a. Add 3.5g (0.3M final concentration) NaCl to each supernatant tube, swirl to completely dissolve.
    b. Add 20g (10% w/v final concentration) PEG 8000 to each supernatant tube, swirl to completely dissolve.
5. Calibrate pH meter
6. Clean probe with copious amounts of 70% ethanol followed by autoclaved Type I water.
7. Test pH and make sure it is between 7.2 and 7.4. Use filter sterilized 6N HCl or 1N NaOH if needed.
8. Prepare an autoclaved Type I water blank following steps 2 through 6.
9. Shake/incubate samples overnight at 4°C and 125-150rpm

Day 2

1. Gather materials:
    - 40 uL of 10^-4 G-block
    - Bleach

- Ethanol
- Absorbent diapers
- Alcohol burner
- Sterilized Pasteur pipets
- Sterilized 1.5mL microcentrifuge tubes

2. Balance and centrifuge the PEG tubes at 4,200rpm (5,020xg) for 45min at 4°C
    a. Use the Beckman-Coulter JS rotor 4.2
3. Carefully aspirate the supernatant to the elbow of the bottle so as not to disturb the pellet (down to the elbow of the tube)
4. Balance tubes and centrifuge the remaining PEG pellet at 2,600rpm (1,500xg) for 5min at 4°C
5. Aspirate all traces of fluid without disturbing the pellet. (Tilt tube and aspirate liquid from the elbow). Compare size against Crypto oil references.
6. Let the pellets warm-up, and flick until the little remaining liquid allows the pellet to become a viscous fluid consistency. **For 2 different samples add 10 uL of 10^-4 G-block.**
7. Use a glass 5 mL pipet to transfer about 0.5 to 0.7 mL of pellet to MoBio Power Soil tubes. Splitting pellet between multiple tubes depending on size.
8. Use a last tube to pipet some of the lysis buffer into the centrifuge tube to wash and wash the inside of the pipet by bubbling.
9. Freeze at -80°C for at least one hour, but up to several weeks to months if needed. Proceed to nucleic acid extraction and purification
10. After extraction: **For the remaining 2 samples add 10 uL of 10^-4 G-block directly to MoBio Power Soil tubes after extraction.**

DNA Extraction from PEG Pellet Using Zymo ZR Soil Microbe DNA Kit for Clean-up

Last Revised: June 2013

Sample Preparation

1. PEG sample as usual.
2. Use pipet and split pellet into multiple ZR BashingBead Lysis Tube (lot# _____). Add **no more than** 500 µL pellet to each tube.
3. Pipette 750 µL of Lysis Solution (lot# _____) into the 250 mL centrifuge tube. Mix and pipet up and down. Add to first ZR BashingBead Lysis Tube. Repeat for each additional ZR BashingBead Lysis Tube.
4. Transfer ZR BashingBead Lysis Tube(s) to -80°C freezer for at least 1 hour, preferably overnight.

Water Microbiology Laboratory Membrane Filtration Clean-up Procedure

1. Place filter towers in UV box for 2 minutes before placing them in wash bin.
2. Change gloves. Wipe gloves with diapers saturated with 10% bleach/water solution, followed by Eliminase/DNA Away wipes, followed by 70% ethanol solution. Wipe all work areas following the same procedure.

DNA Extraction Procedure (Water Microbiology Laboratory)

1. Put on gloves. Wipe gloves with diapers saturated with 10% bleach/water solution, followed by Eliminase/DNA Away wipes, followed by 70% ethanol solution. Wipe all work areas following the same procedure.
2. Remove ZR BashingBead Lysis Tubes from -80°C freezer and thaw to room temperature. This step may be skipped if sample analysis must be expedited.
3. Load ZR BashingBead Lysis Tubes into the bead beater (balanced) and bead beat on "mix" setting for 5 minutes.
4. Transfer the ZR BashingBead Lysis Tubes from the bead beater to a 2 mL tube rack and place in plastic ice container with ice.

Water Microbiology Laboratory DNA Extraction Clean-up Procedure

1. Change gloves. Wipe gloves with diapers saturated with 10% bleach/water solution, followed by Eliminase/DNA Away wipes, followed by 70% ethanol solution. Wipe all work areas following the same procedure.
2. Aseptically transfer ZR BashingBead Lysis Tubes (in 2 mL tube rack on ice) to Level 2.

DNA Extraction Procedure (Level 2)

1. Put on gloves. Wipe gloves with diapers saturated with 10% bleach/water solution, followed by Eliminase/DNA Away wipes, followed by 70% ethanol solution. Wipe all work areas following the same procedure.
2. Place all necessary materials on new Benchkote.
3. Centrifuge (balanced) ZR BashingBead Lysis Tube(s) @ 10,000 x g* @ 20°C for 1 minute.
4. Snap off base of a Zymo-Spin IV Spin Filter (orange cap for liquid samples; lot# _____) and place in Zymo Collection Tube, set-up **one for each ZR BashingBead Lysis Tube**. Pipette up to 400 µL of supernatant to Zymo-Spin IV Spin Filter contained in Collection Tube(s) and centrifuge (balanced) @ 7,000 x g* @ 20°C for 1 minute. Repeat until all supernatant is passed through a single filter.
5. Pipette Soil DNA Binding Buffer (lot# _____) to filtrate in Collection Tube(s) containing sample at a ratio of 3µL binding buffer:1µL sample.
6. Recombine sample at this step, although you might need to use two Zymo-Spin IIC Column if clogging is an issue. Pipette solution up and down a few times. Transfer 800 µL of sample mixture to Zymo-Spin IIC Column (lot# _____) in a new Collection Tubes(s) and centrifuge (balanced) @ 10,000 x g* @ 20°C for 1 minute.
7. Discard flow through from Collection Tube(s).
8. Repeat steps 6 & 7 (*i.e.* transfer 800 µL of sample mixture in initial Collection Tube and centrifuge (balanced) @ 10,000 x g* @ 20°C for 1 minute and then discard flow through). Repeat until all the liquid has been added to the Zymo-Spin IIC Column.
9. Transfer Zymo-Spin IIC Column to a new Collection Tube, pipette 200 µL of DNA Pre-Wash Buffer (lot# _____) to top of column and centrifuge Collection Tube(s) (balanced) @ 10,000 x g* @ 20°C for 1 minute. Discard flow through.
10. Pipette 500 µL of Soil DNA Wash Buffer to each Zymo-Spin IIC Column and centrifuge (balanced) @ 10,000 x g* @ 20°C for 1 minute. Discard flow through.
11. Transfer each Zymo-Spin IIC Column to clean 1.5 mL lo-bind microcentrifuge tube and add 100 µL DNA Elution Buffer (lot# _____) directly to the column matrix (center area of Zymo-Spin IIC Column). Centrifuge (balanced) @ 10,000 x g* @ 20°C for 30 seconds to elute the DNA. Place extract (now ready for PCR analysis) on ice. Discard Zymo-Spin IIC Column. If you used two Zymo-Spin IIC Columns, add 50 µL to the first column and let sit for 5 minutes, spin, discard, add second column to collection tube and repeat. You should collect 100 µL eluate per sample.
12. Clearly label additional extract and archive by freezing at -20°C Level 2 freezer.

Level 2 DNA Extraction Clean-up Procedure

1. Discard Benchkote.
2. Change gloves. Wipe gloves with diapers saturated with 10% bleach/water solution, followed by Eliminase/DNA Away wipes, followed by 70% ethanol solution. Wipe all work areas following the same procedure.

MoBio Power Soil Nucleic Acid Extraction and Clean-up SOP

Version: January 27, 2011

Revised: October 10, 2011; September 29, 2014; October 13, 2014

Always move from Level 1, to 2, to 3. Do not return to lower numbered area until showered and dressed in freshly laundered clothes.

Nucleic Acid Extraction

Gather Supplies (day before if possible) in Culture Lab:

- Sterile 1.5mL microcentrifuge tubes
- Microcentrifuge tube rack
- Finnpipette tips of various sizes
- Ice if more than one extraction is taking place (the samples can be processed on the benchtop, but it is not wise to let extracted DNA sit at room temperature)
- Clean paper spill mat
- Vortex Genie
- Microcentrifuge
- MoBio PowerBead tubes
- Absorbent diapers
- 5% Bleach solution
- 70% Ethanol
- DNA Away

MoBio PowerSoil DNA Isolation Kit – follow kit instructions – *for PEG and solid samples*

For PEG

- Quantiatively transfer all PEG pellet to PowerBead tube(s) (approximately one tube per 750 uL).

For Feces

- Add 0.25g feces to sterile 2mL tubes.

All PowerBead tubes containing sample

- Vortex 2mL tube with feces/bead/lysis buffer mixture, add 60uL of Solution C1 and invert several times
- Secure in the bead beater fitted with a 2mL tube holder assembly (e.g. Disruptor Genie) and process at “mix” speed for 10 min.

Proceed to Level 2 and continue at step 6 of the **MoBio PowerSoil kit Experienced User Protocol** (see below; steps also written in the TTV Bench sheet)

Nucleic Acid Clean-up

Gather Supplies (day before) in Level 2:

- MoBio kit solutions, tubes and spin filters
- Jars of extra 2 and 1.5 mL tubes (autoclaved)
- Boxes of PCR-ready pipet tips (1000 and 100 uL)
- Container for discarded fluid
- Microfuge racks
- Clean paper spill mat
- Level 2 lab coat
- Boxes of appropriately sized gloves
- Absorbent diapers
- 5% Bleach solution
- 70% Ethanol
- DNA Away

1. Centrifuge the PowerBead tubes at 10,000xg for 30sec at room temp, KEEP TUBES LOW TO BENCH WHEN OPENING TO MINIMIZE AEROSOL SPLATTER
2. Transfer supernatant to a clean 2mL collection tube
3. Add 250uL solution C2 and vortex for 5 sec; incubate at 4°C for 5min (could add C2 ahead of time before transfer the supernatant)
4. Centrifuge at room temp for 1min at 10,000xg
5. Transfer no more than 600uL to a clean 2mL collection tube, if more than 600 ul, prepare a second tube
6. Add 200uL of solution C3 and vortex for 5 sec; incubate at 4°C for 5min (if second tube is used in previous step, adjust the volume to maintain 3:1 ratio for the second tube), CHANGE TIPS FOR EVERY SAMPLE
7. Centrifuge at room temp for 1min at 10,000xg
8. Using oversized 2mL tubes, pipet one tube with 1200uL solution C4 for each sample tube (shake to mix solution C4 before pipeting), close caps and open only one at a time while transferring sample
9. Avoid the pellet and transfer up to 750uL supernatant to an oversized 2mL tubes containing C4 and vortex for 5 sec
10. Prepare additional oversized 2mL tubes until all supernatants are transferred
11. Load ~650uL into a clean spin filter and centrifuge at 10,000xg for 1min at room temp. **Combine tubes of the same sample at this step – record in reference table**
12. Discard the flow through and repeat the step 15 until all extract is applied to filter

13. Add 500uL solution C5 to the spin filter and centrifuge at room temp for 30sec at 10,000xg
14. Discard the flow through and centrifuge again at room temp for 1 min at 10,000xg
15. Aseptically transfer the spin filter to a new 2mL collection tube (labeled for long term storage) and add 100uL solution C6 directly to the membrane (incubate at 4°C 5 min. before centrifuging)
16. Centrifuge at room temperature for 30sec at 10,000xg
17. Aseptically discard the Spin filter
18. -The DNA is now suitable for PCR. Save extracts in Box/Ziplock in -20°C freezer (top left drawer of freezer)

qPCR Environmental Master Mix

for Bovine Adenvirus 10

For primers aliquoted 9/15/16
For probes aliquoted 10/7/16

Date:
Investigator:

Reagents	Conc. per rxn tube (µM)	Vol. per rxn tube (µL)	Total volume of reagents(µL)	Master Mix # of tubes
Add H_2O to get 20 (µL)		7.8	0.0	0
Amount of DNA Template		**5.0**	0.0	
TaqMan Env. MM		15.0	0.0	
Forward Primer 10 µM stock	0.3	0.90	0.00	30 uL aliquot
Reverse Primer 10 µM stock (Orange)	0.3	0.90	0.00	30 uL aliquot
Probe 15.3 µM stock	**0.2**	0.39	0.00	35 uL aliquot
Total Volume		30	0.00	
Dispense to each well			**25**	

Step	Time	Temp ºC
DNA polymerase activation	10 min	95
Denaturation	15 sec	95
Anneal	40 sec	58
Denature	1 sec	72
Extend	60 sec	60
Cycle step 2-5	45	

Forward B10F: TTACGCCCAACTTCCTTTTG
Reverse B10R: CCACGCGTCTACTCCGTATT
BoAdV10 P451: 5HEX/ACGAGTTGC/ZEN/GGCTGATCCAATTTATCA/3IABkFQ

Sibley/Pedersen/Zeeb

qPCR Environmental Master Mix

for Porcine Adenovirus

For primers aliquoted 9/15/16
For probe aliquoted 10/7/16

Date:
Investigator:

Reagents	Conc. per rxn tube (µM)	Vol. per rxn tube (µL)	Total volume of reagents(µL)	Master Mix # of tubes
Add H_2O to get 20 (µL)		6.6	0.0	0
Amount of DNA Template		**5.0**	0.0	
TaqMan Env. MM		15.0	0.0	
Forward Primer 20 µM stock	0.9	1.35	0.00	30 uL aliquot
Reverse Primer 20 µM stock	0.9	1.35	0.00	30 uL aliquot
Probe 9.4 µM stock	0.225	0.72	0.00	25 uL aliquot
Total Volume		30	0.00	
Dispense to each well			**25**	

Step	Time	Temp ºC
DNA polymerase activation	10 min	95
Denaturation	15 sec	95
Anneal	20 sec	55
Extend	20 sec	60
Cycle step 2-4	45	

Forward Q-PAdV-F: AACGGCCGCTACTGCAAG
Reverse Q-PAdV-R: GCAGCAGGCTCTTGAGG
Probe Q-PAdV-P: 56-FAM/CACATCCAG/ZEN/GTGCCGC/3IABkFQ

Hundesa 2009

qPCR Environmental Master Mix **Adenovirus A-F Assay**

Date:

Investigator:

Reagents	Conc. per rxn tube	Volume per rxn tube (µl)	Master Mix # of tubes
Add H_2O to make vol of 20 µl		9.55	0
Amount of DNA Template		5	
Environmental MM lot#	exp date	15	0
JTVXP probe Probe (30uM stock)	150 nM	0.15	0
JTVXF forward primer (100 uM stock)	500 nM	0.15	0
mod-JVTXR reverse primer (100 uM stock)	500 nM	0.15	0
Total Volume		30	0
Dispense to each well			25 uL

Primer stock prepared: 8/28/2014 exp. Aug 2017

Probe stock prepared: 8/28/2014 exp. Aug 2017 25 uL aliquots 4/14/17

JTVXR concentrated stock 104.1 uM

Step	Time	Temp ºC
UP Enzyme Activation	10 min	95
Denaturation	10 sec	95
Anneal	30 sec	55
Extend	30 sec	72
Cycle step 3-4	45 cycles total	

Assay modified from Jothikumar 2005

Forward	JTVXF	GGA CGC CTC GGA GTA CCT GAG
modified Reverse	mod-JTVXR	ACI GTG GGG TTT CTR AAC TTG TT
TaqMan Probe	JTVXP	6-FAM-CTG GTG CAG TTC GCC CGT GCC A-BHQ

qPCR Environmental Master Mix

for Bovine Adenovirus 10 and Porcine Adenovirus

Bovine
For primers aliquoted 9/15/16
For probes aliquoted 10/7/16

Porcine
For primers aliquoted 9/15/16
For probe aliquoted 10/7/16

Date:
Investigator:

Reagents	Conc. per rxn tube (µM)	Vol. per rxn tube (µL)	Total volume of reagents(µL)	Master Mix # of tubes	Notes
Add H_2O to get 20 (µL)		4.4	0.0		
Amount of DNA Template		5.0	0.0		
TaqMan Env. MM		15.0	0.0		
Forward Porcine Primer 20 µM stock	0.9	1.35	0.00	30 uL aliquot	
Reverse Porcine Primer 20 µM stock	0.9	1.35	0.00	30 uL aliquot	
Porcine Probe 9.4 µM stock	0.225	0.72	0.00	25 uL aliquot	
Forward Bovine Primer 10 µM stock	0.3	0.90	0.00	30 uL aliquot	
Reverse Bovine Primer 10 µM stock	0.3	0.90	0.00	30 uL aliquot	
Bovine Probe 15.3 µM stock	0.2	0.39	0.00	35 uL aliquot	
Total Volume		30	0.00		
Dispense to each well			**25**		

Number of NFWs	
PCR water for NFWs	
Notes	

Mult-iplex Test

Step	Time	Temp ºC	notes
DNA polymerase activation	10 min	95	
Denaturation	15 sec	95	
Anneal	20 sec	55	
Denature	1 sec	72	
Extend	45 sec	60	
Cycle step 2-5	50		

Bovine

Forward B10F:
TTACGCCCAACTTCCTTTTG
Reverse B10R:
CCACGCGTCTACTCCGTATT
BoAdV10 P451:
5HEX/ACGAGTTGC/ZEN/GGCTGATCCAATTTATCA/3IABkFQ

Porcine

Forward Q-PAdV-F: AACGGCCGCTACTGCAAG
Reverse Q-PAdV-R: GCAGCAGGCTCTTGAGG
Probe Q-PAdV-P: 56-FAM/CACATCCAG/ZEN/GTGCCGC/3IABkFQ

Sibley/Pedersen/Zeeb