A New Field Method for Enumerating Viable *Legionella* And Total Heterotrophic Aerobic Bacteria

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ABSTRACT

A new field method for the quantitative determination of viable *Legionella* and total heterotrophic aerobic bacteria was evaluated in hundreds of laboratory experiments and from analysis of over two thousand field samplers in actual operating conditions. The method has been proven reliable for determining viable cell concentrations of *Legionella pneumophila*, *Legionella* species and total heterotrophic aerobic bacteria. In comparison to standard methods, the new protocol has been shown to be more accurate, faster and more convenient. Comparisons to other rapid methods are presented. Guidance is given for use of the new method within the context of hazard analysis and control to prevent legionellosis.

INTRODUCTION

The patents pending Phigenics Validation Test® (PVT) is a "dipslide format" field sampler that has on its surfaces the standard growth media required for *Legionella* enumeration. The product is designed so that both viable *Legionella* and total heterotrophic aerobic bacteria counts can be obtained from the same test.

More than two thousand PVT field samplers were analyzed in laboratory and field experiments to develop the method, simplify the protocol and compare results to the Standard Method. Laboratory experiments, split sample comparisons and case histories of field use under actual operating conditions document the features and benefits of the PVT.

Features of the PVT

The PVT is a field sampling protocol to obtain viable cell concentrations (as CFU/ml) for the following from the same sample:

- total heterotrophic aerobic bacteria
- *Legionella pneumophila* (serogroup 1)
- *Legionella pneumophila* (serogroup 2-14)
- non-pneumophila *Legionella* species

Data is obtained for the exact moment when the PVT field sampler contacts the water sample

- shipment of water samples to the laboratory is not required
- time required to obtain results is reduced 75-80% compared to the Standard Method for *Legionella*

The PVT field sampler consists of a sterile plastic screw-capped container within which is held a paddle containing on one side buffered charcoal yeast extract agar enriched with α-ketoglutarate (BCYEα) and on the other side of the paddle, BCYEα agar plus the selective supplements glycine, vancomycin, polymyxin B, and cycloheximide (GVPC). Figure 1 shows the PVT field sampler.

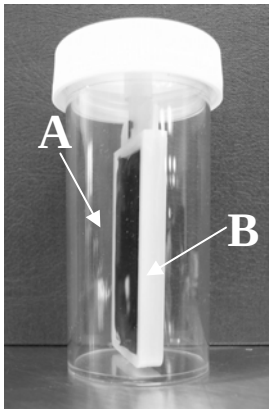


Figure 1. The PVT field sampler consists of a sterile plastic screw-capped container (A) within which is held a paddle (B) containing buffered charcoal yeast extract agar enriched with α-ketoglutarate (BCYEα) on one side and on the other side of the paddle, BCYEα agar plus the selective supplements glycine, vancomycin, polymyxin B, and cycloheximide (GVPC).

Benefits of the PVT

The benefits derived from PVT features are 1) more accuracy, 2) faster turnaround time for results and 3) more convenience compared to the standard methods for *Legionella* and for total bacteria. Compared to the Standard Methods for total bacteria and for *Legionella*, the PVT is:

1. More accurate because variations due to changes in water samples during shipment are entirely eliminated
2. Faster because transit time is not wasted and microcolonies are enumerated directly on the PVT field samplers; typical turnaround time for results is 48-72hrs after sampling
3. More convenient because results for total bacteria AND *Legionella* bacteria are obtained from the same sample with one protocol and results are archived by the Phigenics Analytical Services Laboratory (PASL) in a standardized format for future reference

THE NEED FOR THIS NEW METHOD

Enumerating viable *Legionella* in water samples requires inoculation onto growth medium, confirmation of growth on the medium and confirmation of *Legionella* colonies. All versions of the method relate back to Procedures for the Recovery of *Legionella* from the Environment developed by the Centers for Disease Control and Prevention[1]. The International Standard Organization (ISO) has published ISO 11731 Water quality – Detection and Enumeration of *Legionella* which is generally regarded as the international Standard Method[2].

There are two critical limitations in the Standard Method:

1) the method requires transit of water samples to an analytical laboratory and
2) turnaround time for results is very long.

Typically, water samples are at least 2 days in transit and set-up before the 7-10 day test even begins; time in transit causes significant and unpredictable changes in the sample. Even water samples that are processed a few hours after they have been taken from the system should be regarded as suspect because microbial and chemical factors in water samples are highly dynamic.

Molecular methods to measure *Legionella* in water samples all suffer two critical limitations:

1) inability to differentiate viable from non-viable *Legionella* and
2) no enumeration result, *e.g.*, no viable cells per unit volume sample.

Molecular methods include nucleic acid detection (PCR; polymerase chain reaction or FISH; fluorescence *in situ* hybridization) and serologic methods by antigen/antibody reactions using immuno-specific assays or with differential fluorescent antibody (DFA) direct cell counting. Molecular methods are useful for confirmation of *Legionella* colonies. For the PVT, an immuno-specific method is used to confirm *Legionella* colonies on the surface of paddles.

For water samples in the field, molecular methods are generally not used because of these limitations. A water field method based on FISH requires use of epifluorescence microscopy or an expensive slide scanner in the field and requires a redefinition of "viable" which is inconsistent with how the term is used in the Standard Method.

Even further limited for field use, lateral flow immuno-specific chromatographic devices recently introduced detect only antigens from *Legionella pneumophila* serogroup1. This limitation is unacceptable because there are many other *L. pneumophila* serogroups and other species of *Legionella* which are also potentially hazardous and because presence of any of them indicates conducive conditions in the water system that could be dangerous. During the 1990's, enzyme-linked immuno-specific environmental products with this same limitation were introduced for water analysis but were removed from the marketplace because results were regarded as potentially misleading. Occasionally, a "limit of detection" based on a measure of viability is claimed for these methods (*e.g.*, 100 CFU/ml). This claim is incorrect and misleading since immuno-specific assays cannot distinguish living from dead *Legionella*.

In hospitals today, the most used diagnostic method for presumptive legionellosis is the "urine antigen test". Formats for the method are enzyme-linked or chromatographic immuno-specific assays. Viability assessment and enumeration of *Legionella* are not necessary for this application. The intended use for the urine antigen test is in conjunction with culture and other methods (http://binax.com/NOWlegionella.shtml).

The total heterotrophic aerobic bacterial cell concentration in water is regarded as useful for validation of biological hazard control. For instance, the Association of Water Technologies position paper on legionellosis gives guidance in regard to target concentrations of the total bacteria count[4]. The Cooling Technology Institute also gives such guidance[5].

The PVT is the only field method available that gives results for both *Legionella* and total bacterial counts from the same sample and without a requirement to ship water samples back to an analytical lab (Table 1).

Table 1. Comparison of methods to measure *Legionella* bacteria in water samples.

Feature of the Method	Standard Methods	Molecular Methods[(a)]	PVT
Detects viable bacteria?	YES	NO	YES
Enumeration of viable bacteria in CFU/ml?	YES	NO	YES
Water sample analyzed in the field?	NO	NO (for most methods) [(b)]	YES
Sample spoilage problem eliminated?	NO	NO (for most methods)	YES
Gives total heterotrophic aerobic bacteria?	NO	NO	YES
Typical turnaround time for results?	10-14 Days[(c)]	Minutes - Hours	48-72 Hrs

(a) Molecular methods include polymerase chain reaction, fluorescent *in situ* hybridization, differential fluorescent antibody tests, enzyme linked immuno-specific assays and lateral flow immuno-specific chromatographic assays.
(b) Most methods are not promoted for field use due to inherent limitations. See text above for details
(c) Includes water transit time.

THE PVT FIELD PROTOCOL

The field protocol is fast and simple: 1) label 2 PVT samplers, 2) collect water sample, 3) perform 1st 3s dip, 4) add vial of pH adjust, wait 5min, 5) perform 2nd 3s dip, 6) pack PVT samplers and ship with, 7) inserted in-transit incubators and 8) receive results by email.

Table 2 itemizes PVT protocol steps and instructions.

Table 2. PVT protocol steps and instructions.

Protocol Step	Instructions
1. Establish chain of custody and label 2 PVT field samplers	Complete chain of custody sample submission spreadsheet. Affix completed authenticity labels.
2. Sample collection	Collect 100mls of sample in sterile EPA-approved hinge-capped vial with thiosulfate neutralizing tablet, provided.
3. First PVT dip	Perform 3s dip into the neutralized sample water. Replace PVT sampler into the labeled screw-capped container.
4. Acid treatment	Add the contents of 1 vial solid pH adjust, provided. Allow 5min acid treatment.
5. Second PVT dip	Using the 2nd PVT field sampler, perform a second 3s dip into the same sample vial which has been acid treated for 5 min (step 4). Replace the 2nd PVT sampler into its labeled screw-capped container.
6. Pack PVT samplers for shipment to laboratory	Replace PVT samplers in box, secure, pack into a shipping box with at least 2" packing material on all sides; insert in-transit incubators, provided (see Step 7).
7. Insert in-transit incubation packets into shipping container	Above 80 °F ambient air temperature, use 1 in transit incubator packet per PVT box; below 80 °F ambient air temperature, use 2 in-transit incubator packets per PVT box; ship to analytical lab either overnight or 2 day Air.
8. Receive results report	Receive results by email in standardized report format from the PASL laboratory

COMPARISONS TO STANDARD METHODS

Effect of Water Sample Transit Time

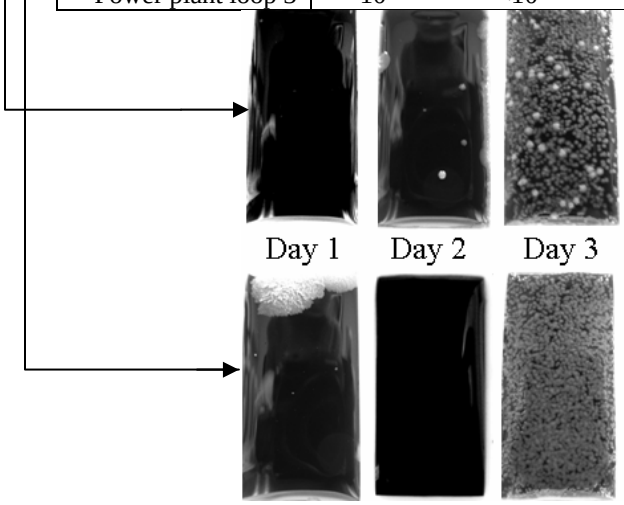
The detrimental effect of water sample holding time on microbial analyses has been known for many years. For instance in one published example, potable water samples had unacceptably high counts of coliform indicator bacteria on the day the samples were taken (Day 1); however, after 24 h storage at 5 °C and 22 °C the levels of viable indicator organisms had declined to such an extent that these same samples met the safe drinking water standard as a result of holding time. In those same water samples, total heterotrophic plate counts increased 0.5 and 2.5 orders of magnitude after 30h and 48h, respectively[3].

The effect of transit time is even more pronounced with more complex water samples such as those from cooling towers.

To enumerate viable *Legionella* using the Standard Method, water samples must be transported to an analytical microbiology laboratory. Unpredictable changes in the microbial and chemical characteristics of water samples can occur. Table 3 and Figure 2 provide examples of holding time effects on water samples.

Table 3. Effect of sample holding time on total heterotrophic aerobic bacteria (CFU/ml) in building potable water samples at ambient temperature (22 °C)

Water Sample	Day 1	Day 2	Day 3
Return loop	10^3	10^6	10^6
Kitchen tap water	10^3	10^2	10^7
Restroom1 tap water	$<10^2$	10^2 - 10^3	10^7
Restroom2 tap water	$<10^2$	$<10^2$	10^7
Power plant loop 1	10^3	10^3	10^7
Power plant loop 2	$<10^2$	$<10^2$	10^3
Power plant loop 3	10^3	$<10^2$	$<10^2$



Several types of water samples were obtained (Figure 2). The tap water samples were inoculated with a mixed culture of bacterial isolates from building water systems. On the day the samples were taken, analyses for *Legionella* and for total heterotrophic aerobic bacteria were performed. After about 24 hours at ambient room temperature (22 °C), the samples were analyzed again. The same samples were analyzed a third time on day 3 when they were about 48h old. Results show that bacterial counts go up in some samples and down in others quite unpredictably. Photographs of the colonies from the three analyses indicate that the types of

microorganisms recovered vary with time. This is all as one would expect given that the microbial components in water sample are alive, dynamic, adaptable and competitive.

	Day 1		Day 2		Day 3	
Water Sample	*Legionella*	Total count	*Legionella*	Total count	*Legionella*	Total count
Tap Water 1	–	10^5	–	10^2	–	10^2
Tap Water 2	+	10^5	–	10^2	–	10^5
Tap Water 3	+	10^5	–	< 100	–	< 100
River Water	ND	10^3	ND	ND	ND	$>10^7$
Water Heater	ND	10^2	ND	ND	ND	10^7
Copper silver treated water	+	10^4	–	< 100	–	< 100

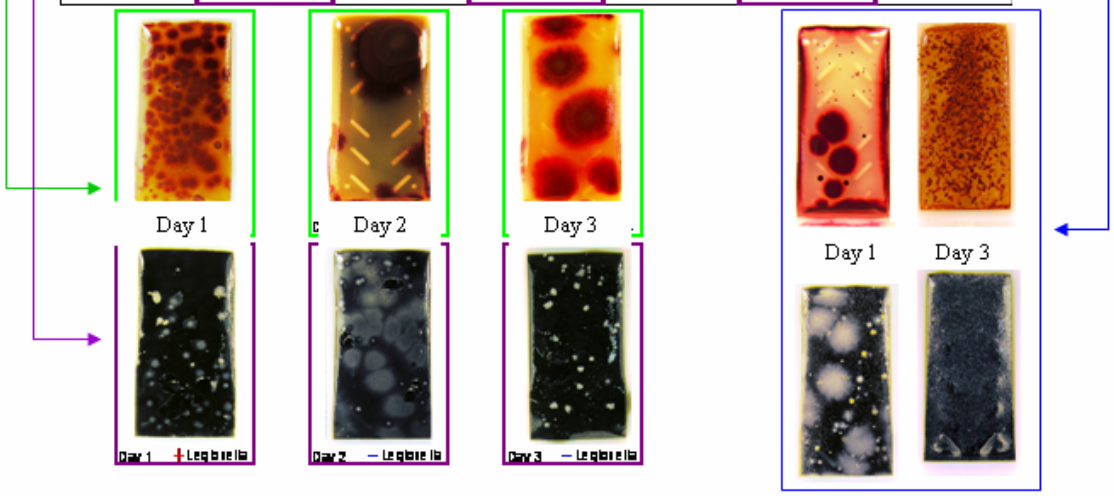


Figure 2. Analyses for *Legionella* and for total heterotrophic aerobic bacteria were performed on the day several types of water samples were taken. Bacterial counts go up in some samples and down in others quite unpredictably. Photographs of the colonies indicate that the types of microorganisms recovered vary with time.

This problem is even more severe in water samples from systems that have been treated with antimicrobials. Oxidizing antimicrobials can be neutralized (*e.g.*, with sodium thiosulfate) to eliminate the negative effect of a sustained contact time on microorganisms; however, this may allow the surviving microorganisms to multiply during transit. Antimicrobials that cannot not be easily neutralized (such as heavy metal ions like copper and silver) are artificially provided a much longer contact time with the microbial components of the sample during water transit compared to what actually occurs in the system; this can cause artificially low results (see Figure 2, "Copper silver treated water", for an example of this effect).

Analysis of aged water samples may result in inaccurate data which could be dangerously misleading.

Split Sample Comparisons of the PVT with Standard Methods

The PVT is similar to the Standard Method in that the same growth media and *Legionella* confirmation steps are used for both. However, the method of sampling is different. For the PVT, the growth medium is dipped directly into the sample; for the Standard Method, an aliquot of sample is removed and spread plated onto the surface of the growth medium. From a physical point of view, spread plating is quite different compared to dipping growth media

directly into the sample. From a hazard analysis perspective, a case can be made that sampling the water with the least physical manipulation of the sample is best; therefore dipping, not spread plating, may be preferred.

Figure 3 illustrates these two methods and shows side-by-side comparisons of 4 examples from a building water system. Results are typically very similar but not identical.

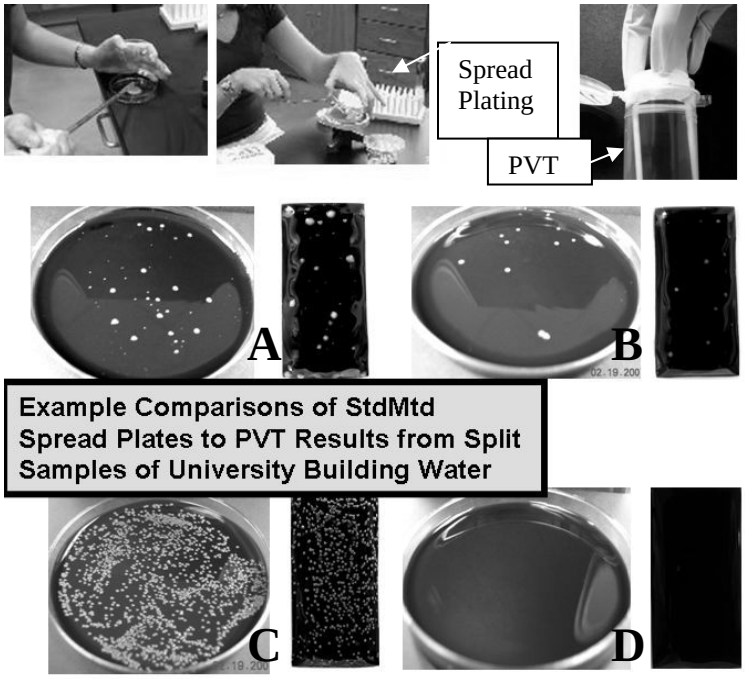


Figure 3. Side-by-side comparisons of spread plating (the Standard Method) and PVT in split samples from a University building water system. Four samples (A - D) are given as examples. Typically, results are very similar but not identical. See Figure 4 for a quantitative comparison from split sample analyzes.

Forty-eight water samples were split in order to compare Standard Method spread plates and PVT analyses from the same University building water system. Figure 4 shows that most (88%) results were not significantly different. However, PVT recovered higher counts in some samples and the spread plate recovered higher counts in other samples. This illustrates that the two methods are similar but not identical.

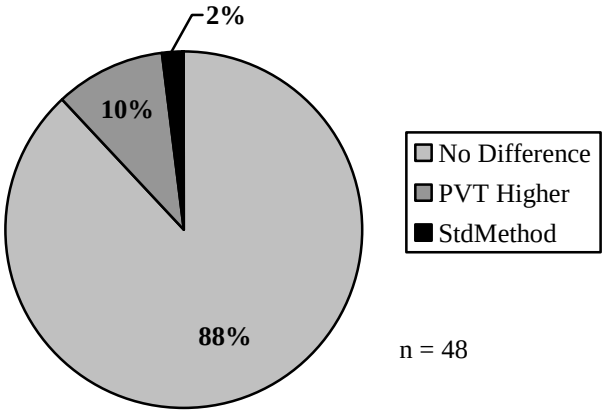


Figure 4. Results from a building water system in which 48 samples were split for comparison with the Standard Method and the PVT.

Split Sample Comparisons of PVT with Total Heterotrophic Aerobic Bacteria Dipslides
Results from split sample analyses with another total heterotrophic aerobic bacteria dipslide system and with the PVT show that results were very similar over a wide range of concentrations (Figure 5). This shows that PVT field samplers are useful for estimating the total bacterial count using order-of-magnitude comparator charts.

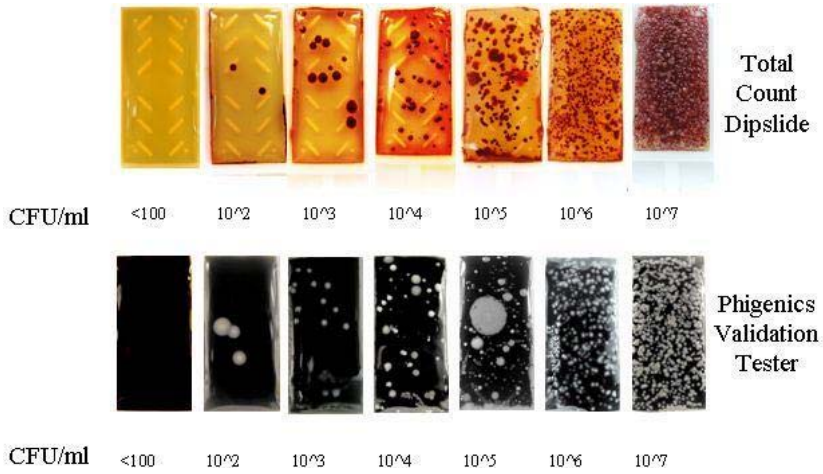


Figure 5. PVT field samplers are useful for estimating the total bacterial count using an order-of-magnitude comparator chart.

Blinded Split Sample Comparisons and Preliminary Statistical Analysis
Blinded comparisons of 147 samples split for analysis with PVT and with the *Legionella* Standard Method were processed by an independent laboratory with no affiliation to Phigenics, LLC. Water transit time variations were not a factor in this study because the lab processed both PVT and samples for the Standard Method. Most (81%) *Legionella* detection results were the same. In 6 % of samples, PVT detected *Legionella* but the Standard Method did not; in 12 % of samples, PVT did not detect *Legionella* but the Standard Method did detect it. In 1 % of samples, *Legionella* was detected by both methods but different species or different serogroups of *L. pneumophila* were recovered.

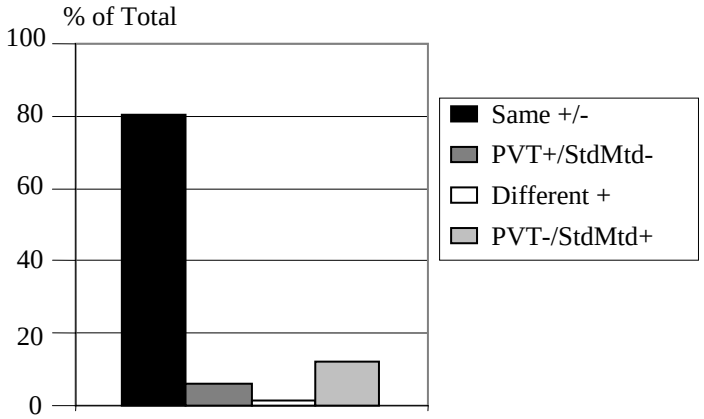


Figure 6. Blinded comparisons of 147 samples split for analysis with PVT and with the *Legionella* Standard Method. Samples were processed by an independent laboratory.

Preliminary Analysis of Accuracy, Specificity, and Sensitivity. These analyses should be considered preliminary because 1) the PVT protocol was being developed and adjusted during the first part of the study (the first 50 samples), 2) the *Legionella* confirmation method was

not the same for both methods (DFA for the spread plates and latex agglutination for the PVT) and 3) the selective supplements used in the assays were different (CAV for spread plates and GVPC for the PVT). Nevertheless, useful information about split sample comparisons can be derived even from this preliminary analysis. The following binary analysis for accuracy, specificity and sensitivity was set to "True" means equivalent result to the Standard Method.
PVT Accuracy. Accuracy is a measure of conformity to an accepted "true" value given as reference [(True Positives + True Negatives)/n samples]. In this study, accuracy was 81.6%.

PVT Specificity. Specificity measures the probability that the result is negative given that the reference is negative [True Neg/(True Neg + False Pos)]. The higher the specificity, the fewer non-hazardous samples will be incorrectly labeled as hazardous. In this study, specificity was 93%. Hazard analysis and validation methods should be set to minimize the probability of incorrectly identifying a building water sample as potentially hazardous.

PVT Sensitivity. There is always a trade-off between specificity and sensitivity. The more sensitive one makes the method, the more false negative results can be expected. Sensitivity measures the probability that the result is positive given that the reference is positive [True Pos/[True Pos + False Neg]. For this analysis "positive" was set at >100 CFU/ml since all 147 samples in this data set were non-potable: Sensitivity was 46%.

It is important to keep in mind that for the purpose of these preliminary comparative binary analyses, the Standard Method spread plate and enumeration results were taken as "true". However, as has been discussed, there are distinct differences between spreading the sample compared to dipping the growth medium into the sample. A case can be made that the PVT protocol may be the more relevant ("true") sampling method for hazard analysis and validation because less physical manipulation of the sample is required.

THE PVT FIELD PROTOCOL - DETAILS

Chain of Custody and Authenticity Labels

Establishing a chain of custody is important when sampling building water systems for *Legionella* and all other microorganisms because data from these analyses are potentially a matter of public health interest. An excellent resource for information about chain of custody procedures is given at http://www.epa.gov/apti/coc/

Chain of custody is a legal term that refers to the ability to guarantee the identity and integrity of the sample from collection through reporting of analytical test results. General guidelines are:

- Keep the number of people involved in collecting and handling samples and data to a minimum
- Only allow people associated with the project to handle samples and data
- Always document the transfer of samples and data from one person to another on chain-of-custody forms
- Always accompany samples and data with their chain-of-custody forms
- Give samples and data positive identification at all times that is legible and written with permanent ink.

For the PVT protocol, the first step in the chain of custody procedure is to complete the PVT sample submission spreadsheet which gives specific information that will be archived along with results from the analysis. This form will establish and document the transfer of samples from the field to the lab.

The next step is to complete and attach the authenticity label to the PVT sampler. The authenticity label documents the identity of the individual who has taken the sample and processed it in the field.

Figure 7 shows the authenticity label and Figure 8 shows the orientation of the how the label must be affixed onto the outside canister of the PVT field sampler.

Tracking #	Acid?	Date
_____ - ___ Last Name - No.	Y N	___/___/___ D / Mo / Yr
Signature of Collector		

Figure 7. The PVT authenticity label. The Tracking # is selected by the individual who takes the sample and processes it in the field. Two PVT field samplers and labels are required for each sample location: one PVT is used before and one is used after acid treatment (indicate acid, Yes or No). The sample date is required. The signature of the sample collector is required. Permanent indelible ink must be used in filling out authenticity labels. A digital image of the authenticity label is made upon receipt of the sample in the analytical laboratory which is archived with the test result report.

Figure 8. Two PVT field samplers are required for each sample location. Affix the authenticity affidavit labels as shown to the outside canisters which hold the dipslides.

Sample Collection

Collect 100mls water using pre-sterilized hinged-lid EPA-approved vial with thiosulfate neutralizing tablet included with the PVT samplers as received. Water samples may include, for example, cooling towers, condensers, storage tanks, drinking water faucets (*e.g.*, immediate and/or post 30s flush), sink faucets, showers (*e.g.*, immediate and/or post 30s flush), pools, spas, hot water tanks (*e.g.* immediate and post 30s flush) and ice from ice machines Close lid and invert to mix sample. One vial is required for each sample location; two PVT samplers are required for each sample location. There will be two PVT dips into the same 100mls of sample contained in the vial.

First PVT Sample Dip

After the neutralizing tablet has dissolved in the sample, remove a PVT field sampler from the screw-capped container and dip it into the water for 3s (Figure 9). Allow excess water to drain away from the PVT sampler surface and replace it into the PVT screw-capped canister. The

PVT sampler is sterile. Do not touch the surface of the sampler. Minimize the time of exposure to air.

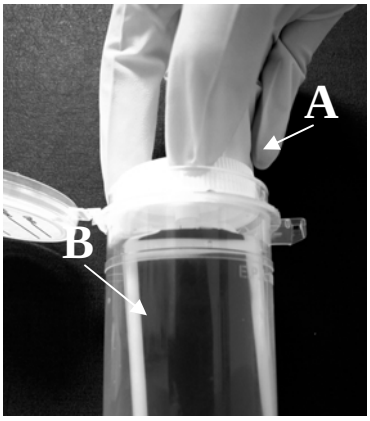


Figure 9. The PVT field sampler (A) is dipped into 100mls of sample contained in the hinged-lid EPA-approved sterile vial (B) that has been filled with 100mls of sampled water.

When the PVT field sampler is dipped into the sample, water is absorbed into each side of the paddle (Table 4). Microorganisms in the water sample are inoculated onto the surface of the growth media as water is absorbed.

Table 4. Replicate trials of PVT samplers dipped for 60s into water (sterile 0.1 mM KCl in distilled water). The PVT sampler paddles absorbed 0.3 ± 0.07 ml of water.

PVT Field Sampler Tested	Water Volume (ml) Absorbed (2 sides)
1	0.290
2	0.284
3	0.448
4	0.250
5	0.222
6	0.229
7	0.229
8	0.199
9	0.323
10	0.294
11	0.332
12	0.139
13	0.308
14	0.262
Ave (Std Dev)	0.272 (0.073)

The volume of water absorbed by PVT field samplers depends upon contact time with the sample (Figure 10). Although the quantity of water absorbed after 60s was about 0.15ml per side, extensive tests with solutions containing microorganisms indicate that the number of viable cells recovered from PVT dips was not significantly different after 3s of immersion (data not shown). The quantity of water absorbed after 3s of immersion was about 0.1ml per side of the paddle (there are two sides to the paddle). Therefore, the limit of detection is based on an estimate of 0.1ml volume absorbed per paddle side after 3s dip. Thus, 1 colony forming unit (CFU) on the paddle surface indicates a viable cell concentration of 1 CFU/0.1ml sample absorbed; this quantity is equivalent to a detection limit of 10 CFU/ml.

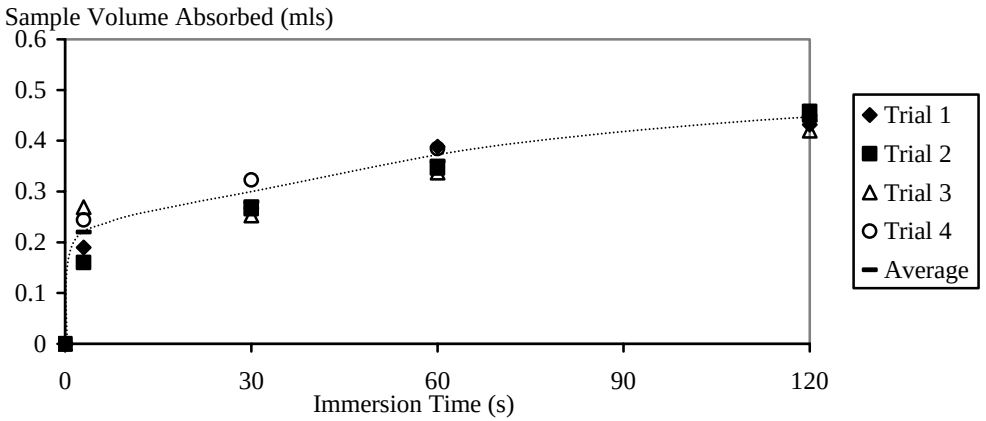


Figure 10. Sample volume absorbed by immersion of PVT field samplers into water depends upon the contact time with the sample. The quantity of water absorbed after 3s of immersion (first sampling time) was about 0.2 ml per paddle (there are two sides to the paddle).

Acid Treatment

Add the contents of the Phigenics pH Adjust vial (included with PVT field samplers) to the 100mls of sample that was just dipped with the first PVT sampler. Close the lid of the container and invert to mix. Wait five (5) minutes; time it.

The purpose of the acid treatment is to inhibit the growth non-legionellae bacteria. Legionellae are more tolerant to acidic pH compared to most naturally occurring waterborne bacteria such as *Pseudomonas, Flavobacterium,* and *Acinetobacter*. Acid treatment therefore helps limit the faster growing competitors that may outgrow *Legionella* on the growth media.

The Standard Method for *Legionella* specifies use of an HCl buffer solution. Comparisons to a proprietary solid phase acid treatment for recovery of bacteria are given in Table 5.

Table 5. Comparison data for bacterial tests with pH Adjust treatments in at least duplicate experiments with controls. Abundant growth (+ + +) after treatment with acid indicates tolerance to the acid treatment.

Microorganism	No Acid Treatment	StdMethod HCl buffer	Phigenics pH Adjust
Legionella pneumophila SG 1	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella pneumophila SG 3	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella pneumophila SG 4	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella pneumophila SG 5	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella pneumophila SG 7	+ +, + + +	+ +, + + +	+ + +, + + +
Legionella anisa	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella bozmanii	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella gormanii	+ + +, + + +	+ + +, + + +	+ + +, + + +
Legionella longbeachae	+ +	+ +	+ +
Legionella micdadei	+ + +, + + +	+ + +, + + +	+ + +, + + +
Pseudomonas aeruginosa	+ + +, + + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Flavobacteium sp.	+ + +, + + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Klebsiella pneumoniae	+ + +, + + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Eschericia coli B	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Enterobacter aerogenes	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Bacillus megaterium	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Bacillus subtilis 301	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Bacillus subtilis SB22	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Non-legionellae isolate - Red	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Non-legionellae - Creamy	+ + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Non-legionellae - Yellow	+ + +, + + +	No Growth (+antibiotics)	No Growth (+antibiotics)
Non-legionellae - Pink	+	No Growth (+antibiotics)	No Growth (+antibiotics)

Extensive side-by-side comparisons of the solid pH adjust and the HCl-buffer specified in the Standard Method were made in samples from real operating building water systems; no significant differences were observed in acid treatments (data not shown). The proprietary solid phase acid pretreatment is preferred because its use is safer and more convenient in the field compared to handling the liquid HCl buffer.

Second PVT Sample Dip

After 5 minutes acid treatment, remove a PVT field sampler from the screw-capped container and dip it into the water for 3s (same as Figure 9). The PVT sampler is sterile. Do not touch the surface of the sampler. Minimize the time of exposure to air. Allow excess water to drain away from the PVT sampler surface and replace it into the PVT screw-capped canister.

Shipping Processed PVT Field Samplers to the Lab

Water samples are processed entirely in the field with the PVT protocol. Water samples are not shipped to the laboratory.

Analysis, data interpretation and archiving of results from field-processed PVT samplers are performed at the analytical laboratory. Therefore, shipping field-processed PVT samplers to the laboratory is required.

A few important precautions are necessary. The growth media on the samplers is perishable. Protect them from freezing or excessive heat. Dropping or jarring the PVT samplers may cause the growth medium to slide off the paddle surface.

After field processing, place the PVT samplers back into the box, secure the box with tape or rubber bands, pack in a box with at least 2” of packing material on all sides, insert in-transit incubator packets (see next section for details), and ship to the lab. Up to 4 PVT boxes containing 40 PVT samplers can be taped or banded together and shipped in one box if at least 2” of packing material surrounds the PVT box assembly. Alternatively, individual PVT samplers can be placed into Styrofoam “peanuts”; up to 30 PVT samplers can be safely shipped in a 14”x14”x14” box (Figure 11).

Figure 11. Individual PVT samplers can be placed into Styrofoam “peanuts”; up to 30 PVT samplers can be safely shipped in a 14”x14”x14” box. Alternatively, place the PVT samplers back into the PVT box they were shipped in (10 PVT samplers per PVT box), secure the PVT box with tape or rubber bands and ship to the lab in with at least 2” of packing material on all sides. Up to 4 PVT boxes can be taped or banded together and shipped in one large box if at least 2” of packing material surrounds the PVT box assembly. Before sealing the box, insert in-transit incubation packets (see Figure 12).

In-transit Incubation

Legionella grows fastest when incubated at about 35 °C. Lower incubation temperatures increase the time required to enumerate colonies and may give competitive advantage to non-legionellae bacteria. In order to reduce the turnaround time for PVT results and to enhance recovery of *Legionella*, an in-transit incubation packet system is used with every shipment of samplers to the laboratory.

In-transit incubators are activated by removing the over-pack and exposing the packet to air (Figure 12). Above 80 °F ambient air temperatures, use 1 in-transit incubator packet per PVT box; below 80 °F ambient air temperatures, use 2 in-transit incubators per PVT box.

Figure 12. In-transit incubators are activated by removing the overpack (A) and exposing the packet to air. Packets are inserted into shipment box packing material and evenly distributed but not closer than 1" to PVT boxes. The packets will stay warm for up to 48h depending on conditions.

Four PVT boxes loaded with samplers were taped together and centered in a large corrugated box filled with Styrofoam peanuts. Eight in-transit incubator packets were evenly dispersed in the peanuts and were at least one inch from each side of the PVT boxes. A thermometer was placed in the very center of the PVT box mass; 5 other thermometers were placed in various locations all around the PVT field samplers inside the shipping box. The experiment was conducted at 22 ºC ambient room temperature (Figure 13).

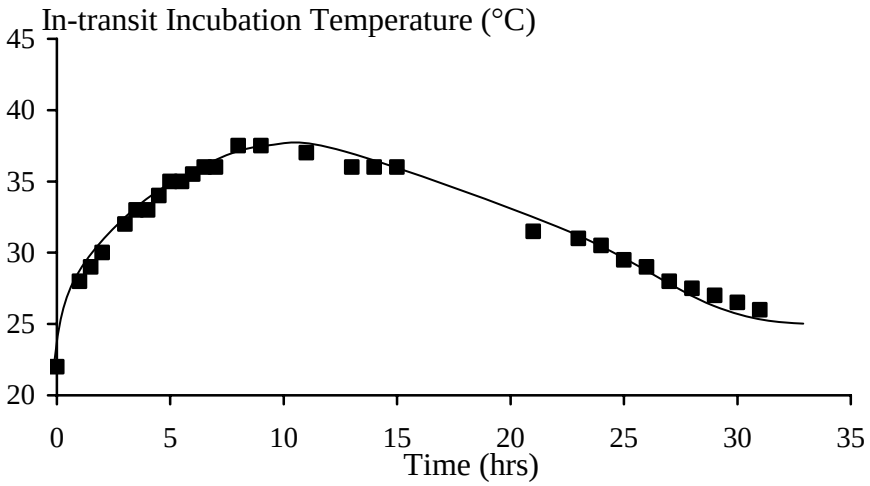


Figure 13. Temperature inside a large corrugated box filled with Styrofoam peanuts. Eight in-transit incubator packets were evenly dispersed throughout and were at least one inch from each side of 4 boxes of PVT samplers taped together and centered in the box. A thermometer was placed in the very center of the PVT boxes; 5 other thermometers were positioned around the PVT boxes. The experiment was conducted at ambient room temperature, 22 ºC. The average temperature of all 6 thermometers around the PVT samplers had a std deviation of about 1.5 °C at each measurement.

Incubators should be spread evenly throughout the packing material at a distance of at least one inch from the PVT boxes or individual samplers. Only 1 incubator per PVT box (10 PVT samplers per box) should be used if ambient air temperatures are at or above 80 °F.

Results Report

Usually, PVT samplers are ready to process when received at the analytical lab; if not, then additional incubation at 35 °C is provided until the sampler is ready to process. Typically, turnaround time for results is 48-72 hours after the samplers are inoculated in the field.

The first step at the lab is to archive digital images of the authenticity labels and all four sides of the PVTs for each sample location (2 PVT samplers are required per sample location).

The total heterotrophic aerobic viable cell count is determined on the paddle side which has not been acid treated and contains no antimicrobial inhibitors in the media. *Legionella*-like colonies on all four paddle surfaces are confirmed with antigen-antibody latex agglutination reagents; the highest *Legionella* count from examination of all surfaces is recorded. *Legionella* colonies are identified on the digital images of PVT surfaces which is included in the results report.

Results are archived in an individual sample report format and also on a summary report which includes the data from the sample submission chain of custody form.

The samplers are usually ready for processing upon receipt at the lab. Every PVT sampler is retained for 10 days; in most cases, results are unchanged at the 10 day recheck (Figure 14). However, some species of *Legionella* grow very slowly. In the small percentage of cases for which significant changes are observed at day 10, an updated report is issued.

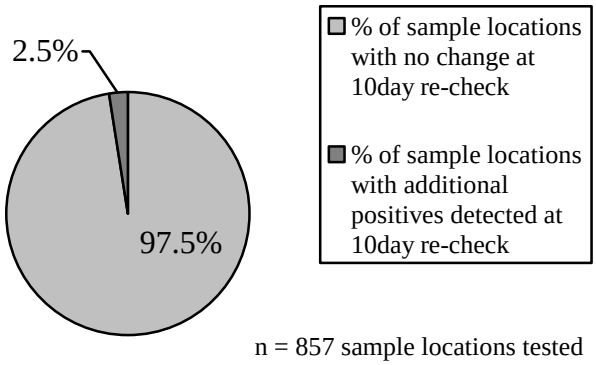


Figure 14. Most (97.5% of 857 sample locations tested) were unchanged after 10 days of incubation. Some species of *Legionella* grow very slowly; in 2.5% of samples analyzed, additional positive *Legionella* detections were made at the 10 day recheck. For those cases, updated result reports are issued.

HOW TO USE PVT RESULTS

The PVT is useful for hazard analysis and for validation of hazard control.

Validation is evidence (data) obtained under operating conditions that hazards have been eliminated or controlled to extent that prevents harm to people.

Eliminating biological hazards or controlling them to an extent that prevents harm to people is the goal for hazard analysis and control.

Preventing Waterborne Disease Associated with Building Water Systems

Prevention of disease from waterborne hazards requires facility managers and owners to answer three simple site-specific hazard analysis and control questions[6]:

- What is the hazard?
- How do we prevent the hazard from harming people?
- How do we know the hazard has been prevented from harming people?

Seven principles comprise effective hazard analysis and control:

- Use process flow diagrams of the water system to perform systematic hazard analysis
- Identify critical control points (process steps at which the hazard can be eliminated or prevented from harming people).
- Establish hazard control critical limits for at each critical control point
- Establish a hazard control monitoring plan for critical limits at critical control points
- Establish hazard control corrective actions for each critical limit
- Establish procedures to document all activities and results
- Establish procedures to confirm that a) the plan actually works under operating conditions (validation), b) is being implemented properly (verification) and c) is periodically reassessed

In practice, a few preliminary steps are necessary. They are:

- Assemble a cross-functional team including at least one person knowledgeable or trained in hazard analysis and control (*e.g.*, a trained employee, or other qualified resource)
- Identify the use and users of the water at the facility to determine at-risk consumers
- Develop process flow diagrams to describe how the product is processed in the facility
- Verify by on-site audit that process flow diagrams are accurate

A typical outline of tasks necessary for the facility team to develop hazard analysis and control Risk Management is:

- TASK 1: Use process flow diagrams to perform systematic hazard analysis of the entire building water system
- TASK 2: Establish validation criteria
- TASK 3: Establish validation and verification schedules and assign management responsibilities
- TASK 4: Using results from TASK 1-3, establish the hazard analysis and control plan.

An Important Note about Corrective Actions and Validation Criteria. Corrective action plans are established to instruct what to do if the hazard control method (*e.g.*, the biocide) is found to be out-of-specification. For instance, a corrective action plan might be: if the free residual chlorine (FRO) measurement is below the lower control limit, increase the product feed rate (for example). Corrective actions are NOT established for what to do if the *Legionella* or total bacteria counts are high. If the validation criteria are violated (*e.g.*, *Legionella* counts are high), it means the hazard control plan is not working under operating conditions. The remedy is to establish a hazard control program that works. This requires systematic hazard analysis. One should be extraordinarily suspicious of recommendations from providers who instruct actions such as “double the biocide dose” in response to a *Legionella* positive result. Such recommendations may be ill-informed, questionably motivated and potentially dangerous.

In February 2007, the World Health Organization (WHO) published a definitive technical consensus opinion entitled *Legionella* and the Prevention of Legionellosis (ISBN 92 4 156297 8)[7]. This work is organized around the Risk Management principles of hazard analysis and control. Every chapter in the book that deals with prevention gives technical details arranged

around the basic principles of hazard analysis and control. These principles apply not only to preventing legionellosis but also, they apply to preventing harm to people from any hazard.

Validation Criteria

Validation is evidence (data) that hazards have been eliminated or controlled to extent that prevents disease under actual operating conditions.

There are no US laws, regulations or standards requiring any particular validation criteria for *Legionella* bacteria or sampling frequency or quantity for monitoring nor is there likely to be absolute rules in new industry Standards. Validation criteria must be established on a site-by-site basis as part of a Water Management Plan. The team developing a Water Management Plan must consult local regulations and guidelines; the following general validation criteria guidelines should not be construed to supercede local or regional guidance. However, based on current guidance and our experience, the following guidelines are generally recommended by Phigenics, LLC:

General Validation Criteria Guidelines

For **potable water systems**, the PVT total bacterial concentration should be ≤1000 CFU/ml and the PVT *Legionella* bacteria concentration should be less than detectable (≤10 CFU/ml). Sampling frequency should be quarterly. In healthcare facilities, take at least two representative samples per 100 beds. For hotels and multi-family residences, take at least two representative samples per floor.

For **utility water systems**, the PVT total bacterial concentration should be ≤10,000 CFU/ml and the PVT *Legionella* concentration should be less than detectable (≤10 CFU/ml). All cooling towers should be sampled quarterly.

The Rationale for Phigenics, LLC Validation Criteria

Potable Water Systems The National Primary Drinking Water Standard (NPDWS) is the federal regulation that defines acceptable microbiological quality. Municipalities are required by law to meet these criteria. The NPDWS criterion for total heterotrophic aerobic bacteria in drinking water is that there should be less than 500 CFU/ml (note: results from microbiological assays are generally not significant unless differences are greater than one order of magnitude; thus, <1000 CFU/ml is not significantly different than 500 CFU/ml). The NPDWS expectation from primary water treatment and disinfection is that there should be no detectable viable *Legionella* in drinking water. That is to say, if drinking water primary treatment is done properly, there should be less than detectable *Legionella* contained in it. Phigenics agrees with those technical experts who state that microbiological quality in building water systems should not be allowed to degrade substantially from the NPDWS criteria. When the municipality delivers water to the consumer, that water becomes the property of the consumer when it cross the water meter and enters the building. The microbiological quality of the water may be degraded in the building as a result of processing in the building water system. It is the responsibility of building facility management to ensure that biological hazards are prevented from harming building occupants and visitors.

Utility Water Systems To our knowledge, the most thorough and most recent survey of viable *Legionella* in US cooling water systems indicates that about 87% of cooling towers have less than detectable (10 CFU/ml) concentrations of *Legionella* in them[8]. That means about 13% of cooling towers in the US should be properly treated to eliminate the hazard, control it or prevent it from harming people. Phigenics believes that there is no valid technical reason for any properly treated cooling water system to have detectable *Legionella* concentrations in the recirculating water. If *Legionella* is detected in cooling water, the hazard should be eliminated through properly applied water treatment. The validation criteria recommended for cooling water is taken from the 2006 position paper published by the Cooling Technology Institute (CTI) in which dipslide results >10,000 CFU/ml are said to indicate that better microbial control should be achieved in the system.

CASE HISTORIES

A Midwestern Chemical Manufacturing Plant Validates Biological Hazard Control
A Director of Operations at a Chemical Plant wanted to validate the success of their recently modified cooling tower biocide program. Previously, he had tested for heterotrophic bacteria, but had not tested for the presence of *Legionella*. Validation testing (validation is evidence that hazards have been controlled under operating conditions) with PVT was performed at two sample locations over 3 months.

Results from PVT validation testing indicated that the new biocide program was effective in controlling heterotrophic microbiological growth and in controlling *Legionella*.

Conclusion: The Director of Operations was pleased with the results that validated the success of the new biocide program. He was pleased with the format of the results report which he has retained for documentation purposes.

Results of PVT Testing Shake Confidence of Industrial Manufacturer in the Pacific Northwest
Facility management at a large Pacific Northwestern manufacturing facility allows a water treatment company to manage their utility water systems including the cooling towers. They were confident, based on written and verbal reports, that their cooling towers were clean and free of any biological hazards. The PVT was used for independent confirmation.

Eight of the twelve (66%) systems tested exceeded the recommended limits for total heterotrophic bacteria, three of them as high as 10^7 CFU/ml, and three of the remaining four were at the upper limits. All but one of the tested systems required some type of immediate remedial action.

The water treatment company providing the outsourced services had been cutting back products and services in an effort to control their own costs. There was not, however, adequate validation (evidence that hazards have been eliminated or controlled under operating conditions) that after cut backs in products and services, the program was still effective.

Conclusions: Results of PVT demonstrated to facility management that improvements in the water treatment program were necessary and that an overall water management plan would be helpful.

PVT Prompts Hazard Analysis and Control at a University on the West Coast
A major university on the west coast employs three different Directors to oversee various parts of the utility and domestic water systems on campus. One of the Directors decided to use the PVT to get a "snap shot" (*i.e.*, hazard analysis) of utility and domestic water at the facility. He was concerned about the potential for biological hazards in the system.

Samples from 10 potable and 3 utility water (cooling tower) systems were analyzed. Results indicated that 9 of 13 systems (69%) had bacterial concentrations higher than recommended levels. Only one of the three cooling towers was in biological control according to criteria set by the Cooling Technology Institute (10^4 CFU/ml of total aerobic bacteria or less), one was at 10^7 CFU/ml and one system exceeded the upper limit of detection (>10^7 CFU/ml) indicating that better microbial control was immediately necessary.

The domestic water tests were even more concerning to the Director with 7 of 10 sample results at or above the upper limits for bacteria in domestic water.
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Conclusions: These results prompted the Director to undertake a systematic analysis of his entire building water system which he intends to use in the development of a Water Management Plan. By this means, he intends to ensure that his facility provides hazard-free water to building occupants and visitors.

Comparison of PVT and Standard Method at a Large Hospital on the East Coast
For many years, building water samples from a large east coast hospital have been taken quarterly for *Legionella* analyses with the Standard Method. The Standard Method analyses are performed at the hospital in the clinical laboratory.

Water samples from nineteen rooms from the same building were split in order to run PVT and Std Method samples in side-by-side comparisons.

According to both methods, tap water in 12 of the 19 rooms had less than detectable viable *Legionella*. According to both methods, tap water in one room had extremely high viable *Legionella* counts (>1000 CFU/ml). The PVT total heterotrophic count (the hospital method does not yield a total bacterial result) was extremely high (10,000 CFU/ml) in this room which further confirmed potentially hazardous conditions. According to both methods, tap water in 3 rooms had very high viable *Legionella* counts (>100 CFU/ml). The remaining 3 rooms had low *Legionella* near detection level according to the Std Method but were negative in the PVT. Conclusion: The PVT and the Std Method equally identified potential biological hazards in the hospital tap water.

Validation in 48h of Successful Disinfection in a Midwestern Hospital Hot Water System

Facility managers at a major Midwestern medical center utilized PVT quick turnaround time to document successful emergency disinfection following a publicly reported case of legionellosis. An administrator at the hospital stated that, although they were already invested heavily in analyzing *Legionella* using other methods, the "benefits of the Phigenics Validation Test did intrigue him." He was interested in both the speed of testing and having more accurate results due to analysis of the water sample immediately after its collection rather than after shipping the water to a lab. Results from PVT analyses were reported back to the hospital 48 hours after samples were processed in the field.

Results indicated that the *Legionella* hazard had been controlled as a result of the disinfection procedure. These results were confirmed by receipt of results from the Standard Method analysis many days later. Typical turnaround time for Standard Method results are 12-14 days (including water sample transit time).

Hazard Analysis and Control for a Residential Shower in the Midwest

A home owner who uses well water was concerned about his residence because of a relatively complex piping system in the master shower and because chlorine disinfectant levels were inconsistent or close to not-detectable in the water. Samples processed on-site with PVT were found to contain extremely high total heterotrophic bacteria (THB) in the supply and dangerously high viable *Legionella* counts (>1000 CFU/ml) in shower water. *Legionella pneumophila*, SG1, SGs2-14 and other *Legionella* species were all detected in the shower water:

	THB (CFU/ml)	*Legionella* (CFU/ml)	FRO (ppm as Cl_2)
Supply	1,000,000	<10	0.37
Heater	100	<10	
Shower	1,000	>1,000	0.08*

*the limit of detection is 0.05ppm

These hazard analysis data prompted hazard control changes: 1) the addition of chlorine disinfectant and 2) consistent flushing of the piping in the shower to compensate for dead legs and stagnant water. The second set of PVT tests and chlorine residual monitoring indicated improvements:

	THB (CFU/ml)	*Legionella* (CFU/ml)	FRO (ppm as Cl_2)
Supply	<100	<10	0.65
Shower	10,000	<10	0.20

The THB concentration in supply water and viable *Legionella* were non-detectable. However, high levels of THB were still present in the shower water. This may indicate biofilms were being removed from surfaces. Conclusion: The homeowner decided to continue both chemical additions and consistent regular flushing until THB in the shower water is shown to be less than 1000 CFU/ml.

Senior Management Opinion about How to Use PVT Results

A major institutional client in the southwestern United States conducted over one hundred PVT analyses and has used the results to better understand the SAFE WATER component of water management plans. The Vice President who was closely involved in looking at the PVT results stated "Being able to use pathogen-related data to best control waterborne hazards is only part of an overall water management plan. We want to know where our needs in this area are and then use appropriate data within a water management plan to have the safest potable and non-potable water possible." He further said "Using data like this is similar to our company doing periodic Employee Satisfaction Surveys. Our senior management uses the results of those Surveys to address and minimize employee dissatisfaction. We then do follow-up Surveys periodically. It's the right thing to do. We don't expect that there will be NO employee dissatisfaction. We want to know the results to best respond to our employees' needs. Similarly, we will use PVT results within a Water Management Plan to best control the waterborne pathogen hazards. We hope but don't expect that there will ever be zero waterborne hazards. Nonetheless, we want to know the PVT results to best control those hazards and provide the safest water possible for our employees and guests."

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