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Standard Guide for Quantification of Microbial Contamination in Liquid Fuels and Fuel-Associated Water by Quantitative Polymerase Chain Reaction (qPCR)[1]

This standard is issued under the fixed designation D8412; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide covers procedures for using quantitative polymerase chain reaction (qPCR), a genomic tool, to detect, characterize and quantify nucleic acids associated with microbial DNA present in liquid fuels and fuel-associated water samples.

1.1.1 Water samples that may be used in testing include, but are not limited to, water associated with crude oil or liquid fuels in storage tanks, fuel tanks, or pipelines.

1.1.2 While the intent of this guide is to focus on the analysis of fuel-associated samples, the procedures described here are also relevant to the analysis of water used in hydrotesting of pipes and equipment, water injected into geological formations to maintain pressure and/or facilitate the recovery of hydrocarbons in oil and gas recovery, water co-produced during the production of oil and gas, water in fire protection sprinkler systems, potable water, industrial process water, and wastewater.

1.1.3 To test a fuel sample, the live and recently dead microorganisms must be separated from the fuel phase which can include any DNA fragments by using one of various methods such as filtration or any other microbial capturing methods.

1.1.4 Some of the protocol steps are universally required and are indicated by the use of the word *must*. Other protocol steps are testing-objective dependent. At those process steps, options are offered and the basis for choosing among them are explained.

1.2 The guide describes the application of quantitative polymerase chain reaction (qPCR) technology to determine total bioburden or total microbial population present in fuel-associated samples using universal primers that allow for the quantification of 16S and 18S ribosomal RNA genes that are present in all prokaryotes (that is, bacteria and archaea) and eucaryotes (that is, mold and yeast collectively termed fungi), respectively.

1.3 This guide describes laboratory protocols. As described in Practice D7464, the qualitative and quantitative relationship between the laboratory results and actual microbial communities in the systems from which samples are collected is affected by the time delay and handling conditions between the time of sampling and time that testing is initiated.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard with the exception of the concept unit of gene copies/mL (that is, 16S or 18S gene copies/mL) to indicate the starting concentration of microbial DNA for the intended microbial targets (that is, bacteria, archaea, fungi).

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.6 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants

D6469 Guide for Microbial Contamination in Fuels and Fuel Systems

D6974 Practice for Enumeration of Viable Bacteria and

[1] This guide is under the jurisdiction of ASTM Committee D02 on Petroleum Products, Liquid Fuels, and Lubricants and is the direct responsibility of Subcommittee D02.14 on Stability, Cleanliness and Compatibility of Liquid Fuels.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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Fungi in Liquid Fuels—Filtration and Culture Procedures
D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this guide, refer to Terminologies D1129, D4175, and the ASTM Online Terminology Dictionary of Engineering Science and Technology.

3.1.2 *amplicon, n*—the product of the qPCR reaction resulting from the amplification of a genetic target using a particular pair of primers.

3.1.3 *gene copies/mL, n*—the unit of concentration for the qPCR assay, indicating the starting concentration of microbial DNA for the intended microbial targets (that is, bacteria, archaea, fungi).

3.1.3.1 *Discussion*—The 16S RNA gene is detected in bacteria and archaea, and the 18S RNA gene is detected in fungi. Both 16S and 18S RNA genes are conserved in prokaryotes and eukaryotes, respectively.

3.1.4 *intercalating dye-based qPCR, n*—qPCR reaction that uses a fluorescent DNA intercalating dye for detection and quantification of amplicon.

3.1.4.1 *Discussion*—Most common intercalating dyes are comprised of asymmetrical cyanine dyes. DNA intercalating dyes are not used in probe-based qPCR.

3.1.5 *molarity (M), n*—moles of solute per L of solution.

3.1.5.1 *Discussion*—1 M = 10^6 µM.

3.1.6 *nanomole (nmole), n*—one billionth of a mole.

3.1.6.1 *Discussion*—1 mole = 6.022×10^{23} molecules and 1 nanomole = 10^{-9} moles.

3.1.7 *oligonucleotide (oligo), n—in qPCR testing*, this refers to the polynucleotide sequence of primers and probes.

3.1.8 *polymerase chain reaction (PCR), n*—an *in vitro* laboratory method for the enzymatic amplification of nucleic acid sequences.

3.1.8.1 *Discussion*—Two DNA oligonucleotide primers anneal with their complementary DNA strands and flank (that is, border) the segment to be amplified. The increase in amount (amplification) of the DNA segments occurs during repeated cycles consisting of three steps: heat denaturation of the double-stranded DNA, cooling to effect annealing of the primers to their complementary DNA strands, and enzymatic extension of the annealed primers by DNA polymerase at its optimal temperature. The repeated cycling of the three steps results in a near exponential increase in the amount of amplicon as defined by the primers.

3.1.9 *primers, n*—oligonucleotides with sequence specificity for the microbial target gene, which is used to initiate the polymerase chain reaction to produce amplicons.

3.1.9.1 *Discussion*—The primers anneal with their complementary DNA strands and flank the target nucleic acid sequence.

3.1.10 *probe-based qPCR, n*—a type of qPCR reaction relaying in the use of fluorescent probes which allows detection of single or multiple targets (that is, multiplexing) in a single qPCR reaction.

3.1.11 *quantitative PCR (qPCR), n*—a PCR-based assay in which the amount of the target DNA sequence present in a specimen can be detected, characterized, and quantified by measuring fluorescence from an intercalating dye or fluorescent oligonucleotide probe.

3.1.11.1 *Discussion*—There are multiple specific techniques by which qPCR can be accomplished but in general the amount of the target DNA sequence present influences the kinetics of amplification and the number of amplification cycles required for the PCR reaction to produce a concentration of amplified products (that is, amplicon) that exceeds a specified threshold, which is determined and used to calculate the number of copies of the target DNA sequence that was present in the sample.

3.1.12 *singleplex, adj—in genomic testing*, indicates that a single target sequence of either DNA or RNA is to be detected.

3.1.12.1 *Discussion*—Singleplex PCR is distinguished from multiplex PCR in which multiple target sequences of either DNA or RNA are detected simultaneously.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *absolute quantification, n—in PCR testing*, the number of gene copies/mL derived from observed specimen counts as a function of observed reference standard counts (see **standards**).

3.2.2 *humic acids, n*—water-soluble substances found in soil and other environmental matrices with the potential to inhibit the polymerase chain reaction process.

3.2.3 *no template control (NTC), n*—a negative control reaction not containing DNA, which is used to detect and determine background contamination or cross contamination.

3.2.4 *probes, n*—oligonucleotides with sequence specificity for the microbial target gene that are terminally labeled with a fluorophore and quencher molecule to detect and quantify the amplicon; probes are required in probe-based qPCR assays.

3.2.5 *recently dead, adj—in microbiology*, a microorganism that is still structurally intact although it is metabolically inactive and not susceptible to resuscitation.

3.2.5.1 *Discussion*—Current methods available for separating whole cells from sample metrices cannot separate live from dead cells.

3.2.5.2 *Discussion*—Although many cells lyse immediately upon death, a percentage of cells can remain intact for prolonged periods after death.

3.2.5.3 *Discussion*—The inability to separate live cells from ghosts makes it impossible for genomic tests to differentiate between analytes extracted from live and ghost cells. Consequently, genomic data are inclusive of live and recently dead cells.

3.2.6 *standards, n*—a DNA sequence spanning the full length of the amplicon, which is serially diluted and used to produce a standard curve for absolute quantification of amplicon in qPCR.

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4. Summary of Guide

4.1 A sample comprising a sufficient volume of fuel or fuel-associated water is collected and processed to provide the genomic DNA required for qPCR testing.

4.2 The DNA sample is tested by performing singleplex or multiplex qPCR. Singleplex is performed by using the primer set for a specific microbial target with a DNA intercalating fluorescent dye or a corresponding fluorescent hydrolysis probe (that is, probe). Multiplex qPCR is performed by combining multiple primer and probe sets in a single reaction (that is, single well) to detect multiple microbial targets simultaneously.

NOTE 1—The principle of the fluorescent hydrolysis probe relies on the degradation of the probe by the 5' to 3' exonuclease activity of the polymerase, which allows the release of the fluorophore from the probe producing the fluorescence.

NOTE 2—In order to detect and accurately quantify any possible bacteria, fungi and archaea in a sample, qPCR assays must be designed with forward (FW) and reverse (RV) primers that are 100 % complementary to the target DNA by using primers and probes with degenerate nucleotides for polymorphic alleles.

4.3 The absolute quantification in gene copies/mL is recorded and analyzed to determine the initial microbial load in the sample.

5. Significance and Use

5.1 This guide provides a protocol for detecting, characterizing, and quantifying nucleic acids (that is, DNA) of living and recently dead microorganisms in fuels and fuel-associated waters by means of a culture independent qPCR procedure. Microbial contamination is inferred when elevated DNA levels are detected in comparison to the expected background DNA level of a clean fuel and fuel system.

5.2 A sequence of protocol steps is required for successful qPCR testing.

5.2.1 Quantitative detection of microorganisms depends on the DNA-extraction protocol and selection of appropriate oligonucleotide primers.

5.2.2 The preferred DNA extraction protocol depends on the type of microorganism present in the sample and potential impurities that could interfere with the subsequent qPCR reaction.

5.2.3 Primers vary in their specificity. Some 16S and 18S RNA gene regions present in the DNA of prokaryotic and eukaryotic microorganisms appear to have been conserved throughout evolution and thus provide a reliable and repeatable target for gene amplification and detection. Amplicons targeting these conserved nucleotide sequences are useful for quantifying total population densities. Other target DNA regions are specific to a metabolic class (for example, sulfate reducing bacteria) or individual taxon (for example, the bacterial species *Pseudomonas aeruginosa*). Primers targeting these unique nucleotide sequences are useful for detecting and quantifying specific microbes or groups of microbes known to be associated with biodeterioration.

5.3 Just as the quantification of microorganisms using microbial growth media employs standardized formulations of growth conditions enabling the meaningful comparison of data from different laboratories (Practice D6974), this guide seeks to provide standardization to detect, characterize, and quantify nucleic acids associated with living and recently dead microorganisms in fuel-associated samples using qPCR.

NOTE 3—Many primers, and primer and probe combinations that are not covered in this guide may be used to perform qPCR. This guide does not attempt to cover all of the possible qPCR assays and does not suggest nor imply that the qPCR assays (that is, combinations of primers and probes, and reaction conditions) discussed here are better suited for qPCR than other qPCR assays not presented here. Additional, primers, primers and probes combination, and qPCR assay conditions may be added in the future to this guide as they become available to the ASTM scientific community. Guide D6469 reviews the types of damage that uncontrolled microbial growth in fuels and fuel systems can cause.

5.4 Culture-based microbiological tests depend on the ability of microbes to proliferate in liquid, solid or semisolid nutrient media, in order for microbes in a sample to be detected.

5.5 There is general consensus among microbiologists that only a fraction of the microbes believed to be present in the environment have been cultured successfully.

5.6 Since the mid-1990s, genetic test methods that do not rely on cultivation have been increasingly favored for the detection and quantification of microorganisms in environmental samples.

5.7 qPCR is a quantitative, culture-independent method that is currently used in the medical, food, and cosmetic industries for the detection and quantification of microorganisms.

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5.8 Since the early 2000s, qPCR methodology has evolved and is now frequently used to quantify microorganisms in fuel-associated samples, but there is currently no standardized methodology for employing qPCR for this application (**1-6**).[3] The purpose of this guide is to provide guidance and standardization for genetic testing of samples using qPCR to quantify total microbial populations present in fuel-associated samples.

5.9 Although this guide focuses on describing recommended protocols for the quantification of total microorganisms present in fuel-associated samples using qPCR, the procedures described here can also be applied to the standardization of qPCR assays for other genetic targets and environmental matrices.

5.10 Genetic techniques have great flexibility so that it is possible to design a nearly infinite number of methods to detect and quantify each and every gene. Because of this flexibility of genetic techniques, it is important to provide a standard protocol for qPCR so that data generated by different laboratories can be compared.

5.11 This guide provides recommendations for primers sequences and experimental methodology for qPCR assays for the quantification of total microorganisms present in fuel-associated samples.

6. Interferences, Special Precautions, and Limitations

6.1 *Introduced Contaminants*—qPCR is a very sensitive technique and the qPCR assays described in this guide will

[3] The boldface numbers in parentheses refer to a list of references at the end of this standard.

detect any bacteria or fungi present in the sample, including those that might be introduced due to improper sample handling during collection, DNA fragments present in the fuel system, sample processing, and testing.

NOTE 4—With the understanding that work in the field cannot be performed under complete aseptic conditions, it is expected that the user of this guide will be trained and become familiarized with microbiological aseptic principles including the use of sterile sample collection bottles, sterile consumables, disinfection of all equipment that cannot be sterilized before use, and use personal equipment (for example, gloves) to prevent introduction of human and environmental microorganisms into the fuel sample that may be detected by the qPCR test.

6.2 *Sample Preservation*—The accuracy and value of the qPCR test is highly dependent on the quality of the sample. The time that passes between the collection of the fuel sample or water, processing (for example, separation of the microorganisms from the fuel and DNA extraction) and testing (that is, performing the qPCR) should be minimized.

NOTE 5—The microbial composition of a fuel sample can change upon retrieval from the fuel system. Thus, it is recommended that samples are processed within a few hours from retrieval or the fuel sample to be refrigerated to prevent changes in microbial composition.

NOTE 6—DNA samples can degrade and factors such as nucleases, temperature, and time may affect the speed and severity of the degradation. DNA preservation techniques including stabilization solutions, refrigeration, freezing, dehydration, lyophilization may be used to mitigate or prevent DNA degradation.

6.3 *Inhibitors of qPCR*—Consideration should be given to the potential presence of polymerase chain reaction inhibitors and other factors in a sample that may affect the qPCR test results. Common inhibitors of qPCR include humic acids and heavy metals. Approaches that mitigate the effect and interference of inhibitors should implemented.

NOTE 7—Multiple commercial DNA extraction kits incorporate strategies to remove potential inhibitors of qPCR from the DNA sample. The use of a DNA extraction protocols that stabilize the DNA sample is recommended.

6.4 *Limitations of qPCR*—qPCR will detect all nucleic acids associated with microorganism whether or not the DNA was from living microbial cells or freely circulating in the fuel sample. For the detection of biocontamination in fuel, it is DNA found inside living microbial cells which is of most importance when assessing the potential for biodeterioration of the fuel. Cells which have recently died (for example due to application of a biocide treatment) can retain DNA within an intact cell structure. However, DNA derived from cells which are long-dead, or which were not involved in recent active microbial proliferation in the system sampled, will generally be present as free-DNA fragments un-associated with cellular material. Thus, it is recommended that strategies that reduce the contribution of free-DNA to qPCR results be implemented.

NOTE 8—Although not yet validated for qPCR analysis of fuels, a filtration process that incorporates a buffer wash step, as described in Practice D6974 for collection of microbial cells from fuels for culture analysis, may be used to collect microbial cells from fuel samples for qPCR analysis. Because free-DNA fragments are too small to be retained by the 0.45 µm membrane filter specified in this guide, only intact cells will be retained. Both living and dead cells could be retained on the membrane filter if they are intact. Consequently, the filtration process will not necessarily eliminate all DNA from dead cells from the qPCR analysis. However, it should eliminate free-DNA derived from cells not involved in recent microbial proliferation. The buffer wash step is expected to further reduce any traces of free-DNA fragments. Alternatively, if the background level of DNA of a given clean fuel system has been ascertained through a proper maintenance sampling regime, then, it is possible to accurately assess increased microbial presence in the fuel without the need to remove all free-DNA from the sample prior to testing.

6.5 *DNA Extraction Efficiency*—A variety of procedures are available for extracting DNA from samples in preparation for qPCR analysis. It is outside the scope of this guide to prescribe the extraction procedure to be used, but it should be recognized that the extraction procedure will have a key bearing on results obtained. Procedures should be used which minimize the loss of DNA during the extraction process. Ideally, the efficiency of DNA extraction will be validated, for example, by simultaneous analysis of the same sample matrix spiked with DNA standards and the use of appropriate negative and positive controls. Extraction efficiency should be demonstrated to be no less than 1 % and ideally better than 5 %. ASTM has a program to develop and update standards relevant to drawing of samples and preparation of samples, including DNA extraction for qPCR analysis.

7. Apparatus

7.1 *Quantitative Real-time PCR Thermocycler,* with the following approximate specifications: ramp rate (≥3 °C⁄s average); temperature (0 °C to 100 °C range, ±0.2 °C accuracy, ±0.4 °C well-to-well uniformity); lid heats up to at least 105 °C; optical detection (able to excite and detect ≥3 multiplexed fluorophores including emission wavelength 517 nm, 556 nm, 615 nm); Scan time for one channel of approximately 3 s; reaction volume from 10 µL to 50 µL; sensitivity of one gene copy of target sequence; dynamic range of ten orders magnitude; provides singleplex and multiplex analysis software for quantification.

7.2 *Micropipetters,* of volumes 1 µL to 10 µL, 10 µL to 100 µL, and 100 µL to 1000 µL.

7.3 *Sterile Laminar Flow Hood, Clean Bench, qPCR Enclosure, or Biosafety Cabinet.*

8. Reagents and Materials

8.1 *Buffer, Tris-EDTA (TE)*—10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0.

8.2 *Master Mix*—Solution containing the required reagents to perform qPCR including a hot-start DNA polymerase, dNTPs, $MgCl_2$, intercalating fluorescent dye, primers, probes, and water.

NOTE 9—To produce the qPCR reaction, commercially available high-quality pre-made master mixes that contain all the components required to perform the qPCR reaction, with the exception of primers, probes, DNA, and water may be used.

8.3 *Micropipette Tips, Plastic,* (filtered micropipette tips are recommended but not required).

8.4 *Primers*—Forward primer and reverse primer for the specific qPCR assays (see Table 1).

NOTE 10—For reliable results, obtain primers from a commercial vendor with a strong track record in primer synthesis.

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TABLE 1 List of Primers, Probes, and Standards used in this Guide

Primer/Probe/Standard	Sequence (5' to 3')	Melting Temperature (°C)	Amplicon length	Reference
FW-16S-Uni (Bacteria)	GTGSTGCAYGGYTGTCGTCA	58		(7, 8)
RV-16S-Uni (Bacteria)	ACGTCRTCCMCACCTTCCTC	58		(7, 8)
16S-Uni-Probe[A] (Bacteria)	5' FB- TGTYGTGARATGTTGGGTTAAGT CCCGYAACGAGCGCA -3' CQM	69		(1)
16S-Uni-Standard[D] (Bacteria)	**GTG**_C_**TGCA**_T_**GG**_C_**TGTCGTCA**GCTCG**TGT**_T_**GTGA** _A_**ATGTTGGGTTAAGTCCCG**_CAACGAGCGCA_ ACC CTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACT CAAAGGAGACTGCCAGTGATAAACTG **GAGGAAGGTG**_G_**GGA**_T_**GACGT**		147 bp	(1)
18S-Uni-FW (Fungi)	GCYYGAATAYATTAGCATGGAATAAYR DAAYAVGA	58		(1)
18S-Uni-RV (Fungi)	CCAAGAATTKCACCTCTGACARYWSAA TACTGA	58		(1)
18S-Uni-Probe[B] (Fungi)	5' FR-TTGTTGGYTTSYARGACCVHCG TAATGATTAATAGGGAYRGTCG-3' CQR	66		(1)
18S-Uni-Standard[D] (Fungi)	**GC**_TC_**GAATA**_C_**ATTAGCATGGAATAA**_TAG_**AA**_T_**A**_G_ **GA** CGTGTGGTTCTATT**TTGTTGG**_T_**TT**_CT_**A**_G_**GACC** _GC_**CGTAATGATTAATAGGGA**_CA_**GTCG**GGGGCA**T** **CAGTATT**_CAAT_**TGTCAGAGGTG**_A_**AATTCTTGG**		132 bp	(1)
Arch-FW (Archaea)	AATTGGCGGGGGAGCAC	58		(9)
Arch-RV (Archaea)	GGCCATGCACCWCCTCTC	58		(9)
Arch-Probe[C] (Archaea)	5'FG-ACAACVSGW/CIQ/RGAGCCTGCGG WTTAAYTGGAYTCAACGC- 3'CQG	68		(1)
Arch-Standard[D] (Archaea)	**AA TTG GCG GGG GAG CAC CAC AAC**_GC_**G**_TG_**G AGC CTG** **CGG**_T_**TT AA**_TTGG AT_**T CAA CGC** CGG ACA TCT CAC CAG GGG CGA CAG CAG AAT GAT GGC CAG GTT GAT GAC CTT GCT TGA CAA GCT **GAG AGG**_A_**GG TGC ATG GCC**		137 bp	(1)

[A] FB, fluorophore of emission peak 517 nm (blue); CQM, compatible quencher blue.
[B] FR, fluorophore of emission peak 615 nm (red), Compatible Quencher (CQR) Red.
[C] Fluorophore of emission peak 556 nm (green), Compatible Quencher Green (CQG); /CIQ/, compatible internal quencher.
[D] Bold characters indicate primers and probe landing region, italicized characters indicate single nucleotide polymorphisms.

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8.5 *Probes*—Fluorescent hydrolysis probes for the 16S and 18S qPCR assays (see Table 1).

NOTE 11—For reliable results, obtain probes from a commercial vendor with a strong track record in probe synthesis.

8.6 *qPCR Plates or qPCR Tubes, Plastic,* specification per the qPCR thermocycler manufacturer.

8.6.1 *Sealing Tape, Optical,* if qPCR plates are used.

8.6.2 *Caps, Optical,* if qPCR tubes are used.

8.7 *Synthetic Oligonucleotide (Oligo) Standards*—The sequence and characteristics of the qPCR oligo standards for the specific assays are presented in Table 1.

NOTE 12—To ensure the quality and properties of the synthetic oligos, they should be ordered with Polyacrylamide Gel Electrophoresis purification (that is, PAGE purified). The oligos should be ordered from a commercial vendor with a strong track record in synthesis of long oligos.

8.8 *Water, Sterile,* ultra-pure water (with a resistivity of 18.2 MΩ.cm) or molecular grade water.

9. Hazards

9.1 Personal protective equipment including laboratory coat, nitrile gloves, and safety glasses are recommended. Additional protective equipment and biosafety procedures may be required depending on the type of microorganism and chemical matrix in use. DNA by itself (that is, naked DNA) is not considered biohazardous.

10. Procedure

10.1 A sufficient liquid fuel sample or fuel-associated water sample should be drawn in accordance with Practice D7464.

NOTE 13—The volume of liquid required depends on the microbial population density in the sample. Typically, 100 mL of fuel or 20 mL of fuel-associated water is sufficient.

10.2 Any microorganisms present in a liquid fuel sample must be separated from the fuel phase prior to performing the qPCR procedure using one of several available methods, which may include a filtration step or a liquid-liquid extraction step.

NOTE 14—Practice D6974 provides a procedure for separation of microorganisms from the fuel by filtration.

NOTE 15—A fast liquid-liquid extraction procedure to recover microorganisms from hydrocarbon fuels (for example, jet fuel) into an aqueous buffer has been shown (**1**).

10.3 Extract the DNA of the microorganisms by using an established total DNA isolation procedure or commercial genomic DNA extraction kit. Store DNA at approximately –20 °C. Alternatively, it is possible to perform qPCR analysis directly on certain types of microorganism suspended in aqueous solutions compatible with qPCR without the need of a formal DNA extraction procedure (**1, 10-12**).

NOTE 16—There are multiple commercial kits that can be used for the extraction of microbial DNA from different environmental samples. There are multiple published protocols for isolation of total microbial DNA, including those presented in the reference book Molecular Cloning: A Laboratory Manual by Sambrook and Russell (**13**). It is recommended that the DNA isolation protocol be designed to deal with environmental samples (for example, soil, water) and take specific steps to remove impurities that may interfere with qPCR such as heavy metals and humic acids. DNA extraction methods that employ a combination of mechanical lysis (for example, bead beating) and chemical lysis appear to be the consensus DNA extraction method in the literature for fuel testing. It is critical that the DNA extraction method used provides efficient cell lysis and DNA recovery for a wide range of bacterial and fungal cells.