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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.

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.