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.

Designation: D8070 – 23

Standard Test Method for Screening of Fuels and Fuel Associated Aqueous Specimens for Microbial Contamination by Lateral Flow Immunoassay[1]

This standard is issued under the fixed designation D8070; 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 test method describes a procedure that can be used in the field or in a laboratory to detect antigens indicative of microbial contamination in liquid fuels, including those blended with synthesized hydrocarbons or biofuels, with kinematic viscosities (at 40 °C) of ≤24 mm^2s^{-1} (for example, Specifications D396, D975, and D1655) and in fuel-associated water.

1.1.1 This test method has been validated by an ILS for a range of middle distillate fuels meeting Specification D1655, EN590, Specification D975, and ISO 8217:2012.

1.2 This test method semi-quantitatively assesses the concentration of specific antigens generated by commonly recovered, fuel-associated, aerobic microorganisms during active growth in fuel systems.

1.2.1 A proprietary formulation of antibodies and antibody mixtures is used to detect three types of microbial antigen contamination: antigens generally found in aerobic bacteria, antigens generally present in common fungi (yeast and molds), and an antigen that is characteristic of *Hormoconis resinae* (the fungus most commonly associated with fuel biodeterioration).

1.2.2 Although the antibodies and antibody mixtures are characteristic of diverse types of bacteria and fungi, it is unlikely that they are universal. Recognizing that for every microbe that has been isolated and characterized, it is likely that there are a billion that have not. Consequently, as is the case with all microbiological test methods, this test method does not purport to detect 100 % of the microbes present in a fuel or fuel-associated water sample.

1.3 For each of the three sets of antigen detected (*H. resinae*, common fungi, and aerobic bacteria), the test detects whether the antigen concentration present is within set ranges representing negligible, moderate, or heavy microbial contamination.

1.3.1 For fuel specimens, the antigen concentration ranges detected are <150 µg/L (negligible), 150 µg/L to 750 µg/L (moderate), and >750 µg/L (heavy).

1.3.2 For specimens of water associated with fuel, the antigen concentration ranges detected are <33 µg/mL (negligible), 33 µg/mL to 166 µg/mL (moderate), and >166 µg/mL (heavy).

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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.* For a specific hazard statement, see Section 8.

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]

D396 Specification for Fuel Oils
D975 Specification for Diesel Fuel
D1129 Terminology Relating to Water
D1655 Specification for Aviation Turbine Fuels
D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants
D4176 Test Method for Free Water and Particulate Contamination in Distillate Fuels (Visual Inspection Procedures)
D6469 Guide for Microbial Contamination in Fuels and Fuel Systems

[1] This test method 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.

*A Summary of Changes section appears at the end of this standard

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D6974 Practice for Enumeration of Viable Bacteria and 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
D7687 Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel and Fuel-associated Water With Sample Concentration by Filtration
D7847 Guide for Interlaboratory Studies for Microbiological Test Methods
D7978 Test Method for Determination of the Viable Aerobic Microbial Content of Fuels and Associated Water—Thixotropic Gel Culture Method
E1326 Guide for Evaluating Non-culture Microbiological Tests
E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *Other Standards:*

BS EN590 Standard for Diesel Fuel[3]
ISO 8217:2012 Petroleum products—Fuels (Class F)—Specifications of marine fuels[4]
NATO Logistics Handbook Chapter 15: Fuels, Oils, Lubricants and Petroleum Handling Equipment, Annex A: Aide Memoire on Fuels in NATO[5]
IATA Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks, Fifth Edition, 2015[6]

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this test method, refer to Terminologies D1129, D4175, E2756, and Guide E1326.

3.2 *Definitions:*

3.2.1 *aerobic, adj*—(*1*) taking place in the presence of oxygen, (*2*) living or active in the presence of oxygen.

3.2.2 *antibody, n*—an immunoglobulin, a protein that is produced as a part of the immune response which is capable of specifically combining with the antigen.

3.2.2.1 *Discussion*—In the context of this test method, antibodies created for this purpose are utilized in conjunction with visual indicators to detect presence of microbial antigens.

3.2.3 *antigen, n*—a substance that stimulates the host to produce an immune response. In the context of this test method, specific antigens are detected as indicators of microbial contamination.

3.2.4 *buffer, n*—a compound or mixture that, when contained in solution, causes the solution to resist change in pH.

3.2.4.1 *Discussion*—Each buffer has a characteristic limited range of pH over which it is effective.

3.2.5 *colony, n*—a discreet visible aggregate of microorganisms that develops when a viable microorganism, or particle containing viable microorganisms, is introduced into a gel-based nutritive culture medium and reproduces there.

3.2.6 *colony forming unit (CFU), n*—a viable microorganism or aggregate of viable microorganisms, which proliferate(s) in a culture medium to produce a viable colony.

3.2.7 *lateral flow device, n—in immunology*, an antibody-impregnated, porous medium through which an antigen-containing buffer is permitted to wick in order to bring the antigen into contact with the antibody.

3.2.7.1 *Discussion*—Typically, the antibody is linked to an indicator which produces a color reaction when antibody and antigen combine.

3.2.8 *metabolite, n*—a chemical substance produced by any of the many complex chemical and physical processes involved in the maintenance of life.

3.2.9 *microorganisms, n*—organisms too small to be seen with the naked eye, which generally include bacteria, protozoa, fungi, and microalgae (sometimes collectively called slime or microbial contamination).

3.2.9.1 *Discussion*—In the context of this test method, microorganisms are bacteria and fungi (yeasts and molds) that are capable of growth in fuels and associated aqueous-phase fluid.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *extraction fluid, n*—a mixture of buffer and surfactants used to extract antigens from the specimens.

3.3.2 *surfactant (surface active agent), n*—a substance that affects the interfacial or surface tension of solutions even when present in very low concentrations.

3.4 *Acronyms:*

3.4.1 CFU—colony forming unit

3.4.2 FSII—fuel system icing inhibitor

3.4.3 LFD—lateral flow device

4. Summary of Test Method

4.1 Microbial contamination is detected using a series of antibodies immobilized onto a test tray in the form of three pairs of lateral flow devices (LFDs). These antibodies are used to detect antigens from common bacteria and fungi that proliferate in fuel tanks and systems.

4.1.1 The LFDs contain broad spectrum antibodies raised against cell components and materials generated during microbial growth on hydrocarbons (the antigens). These antibodies indicate the presence of *H. resinae*, other common fungi, and aerobic bacteria within concentration ranges described in 1.3.

4.2 Fuel or aqueous specimens from fuel are mixed with an aqueous extraction fluid in an extraction bottle. The extraction fluid captures antigenic material present in the specimen.

4.3 Four drops of the separated extraction fluid are dispensed into the sample well of each of the six LFDs, which are arranged in three pairs.

[3] Available from British Standards Institution (BSI), 389 Chiswick High Rd., London W4 4AL, U.K., http://www.bsigroup.com.

[4] Available from International Organization for Standardization (ISO), ISO Central Secretariat, BIBC II, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, http://www.iso.org.

[5] Available from North Atlantic Treaty Organization (NATO) at http://www.nato.int/docu/logi-en/1997/lo-15a.htm.

[6] Available from International Aviation Transport Association (IATA) at http://store.iata.org.

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4.4 For each of the three types of contamination (*H. resinae*, other common fungi, and aerobic bacteria), a pair of LFDs indicates the concentration range of the detected antigen present in the specimen. For each LFD pair, the minimum detection level is set so that one is at the lower boundary of the moderate contamination range and the other at the lower boundary of the heavy contamination range of antigen concentration stated in 1.3.

4.5 Presence of antigen below the minimum level of detection for each LFD is indicated by development of red control and test lines. If only the control line becomes visible, then antigen concentration in the specimen is above the minimum level of detection for the LFD. When antigen is present at or above threshold concentrations, test lines on the LFD fail to appear. By reading both LFDs in the pair, it can be established which range the antigen concentration falls within, that is, negligible, moderate, or heavy.

4.6 The control lines appear within 10 min and the result is read at 15 min, although the lines are stable up to 30 min.

4.7 If contaminating antigens are not present, test lines will also appear within 10 min and the result is read within 30 min (see 4.6). If test lines do not appear, then this indicates the presence of contamination at the concentration ranges described in 1.3.

4.8 The results and antigen concentration ranges are determined and recorded.

5. Significance and Use

5.1 This test method is intended to provide a tool for assessing whether fuel storage and distribution facilities, or end user fuel tanks, are subject to microbial growth, and to alert fuel suppliers or users to the potential for fuel quality or operational problems or the requirement for preventative or remedial measures, or both.

5.2 This test method allows assessment of whether antigens generated by microbial activity in the specimens are present within specific defined ranges.

5.3 This test method measures the presence of microbial and metabolite antigens in a specimen. The antigens are generated from the living cells and metabolites created by fungi and bacteria during growth on fuel. Consequently, the presence of antigens is an indicator of microbial contamination in fuel systems. Antigens are not associated with matter of nonbiological origin.

5.3.1 Some of the antigens detected by this test method can persist after treatment with a biocide. See 11.4.

5.4 This test method is semi-quantitative and can be used to determine whether contamination in samples drawn from fuel tanks and systems is negligible or present at moderate or heavy levels.

5.4.1 Further information on using the test to assess biodeterioration risk is provided in Appendix X1.

5.5 The significance of these levels to the operator will depend on the fuel type, the sampling location, the equipment or facility sampled, and the specific operating circumstances.

5.6 Further guidance on interpretation of test results can be found in Guide D6469, in Energy Institute guidelines for the investigation of the microbial content of petroleum fuels, and in the IATA Guidance Material on Microbial Contamination in Aircraft Fuel Tanks.

5.7 Further guidance on sampling can be found in Practice D7464.

5.8 Testing can be conducted on a routine basis or to investigate incidents.

5.9 Microbiological tests are not intended to be used to determine compliance with fuel specifications or limits. The implementation of specification limits for microbiological contamination in fuels is generally not appropriate, and microbial contamination levels cannot be used alone or directly to make inferences about fuel quality or fitness for use.

5.10 When interpreting results, it must be appreciated that the test result applies only to the specific sample and specimen tested and not necessarily to bulk fuel. Microbiological contamination usually shows a highly heterogeneous distribution in fuel systems, and therefore, analysis of a single sample will rarely provide a complete assessment of the overall levels of contamination present.

5.11 Water phase will usually contain substantially higher amounts of microbial contamination than fuel phase and, consequently, a different interpretation of results is required. This is why this test method reports antigen concentration per mL for water and per L for fuel.

5.12 This test method differs from some other methods (for example Test Methods D7687 and D7978) and practices (for example Practice D6974) in that it detects microbial activity in fuels or associated aqueous specimens in the field and does not need to be performed in a laboratory or in an aseptic environment. It may be used in a laboratory.

5.13 This test method does not require specialist microbiological experience or knowledge.

5.14 This test method provides rapid results that reflect the total active microbial contamination in the specimen, and enables result to be obtained within 15 min.

5.15 This test method differentiates among three ranges of contamination for *H. resinae*, other fungi, and aerobic bacteria (see 1.3).

6. Interferences and Possible Test Method Errors

6.1 Drops of the extraction fluid can fail to be expelled from the extraction bottle if particulate material in the sample blocks the dropper nozzle. The nozzle should be removed and cleared.

6.2 If fuel inadvertently comes into contact with the LFD, it can prevent the control line developing and the test will be invalid. Contact of fuel specimen with the LFD must be avoided.

6.3 Do not touch the LFD viewing windows. Touching the viewing windows can contaminate the LFD. If this occurs, the test is invalid. Repeat steps 10.1 – 10.9

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6.4 Test line color intensity is affected by contamination levels. Above the threshold levels of antigen concentration (that is, above moderate and above heavy), the test line will not appear. However, as these concentrations are approached, the line may be faint. A moderate or heavy level of contamination should not be reported unless the test line is not visible.

6.5 Test lines are visible when target antigens are present at concentrations below the lower thresholds. When antigen concentrations are below detection limits (see 11.3.1), all lines are visible and contamination levels are considered to be negligible.

6.6 Test lines on the high LFDs are visible at concentrations greater than the lower thresholds (>150 µg/L for fuel samples or >33 µg/mL for water sampels) but less than the higher thresholds (≤750 µg/L for fuel samples or ≤166 µg/mL for water samples) (1.3, X1.1).

6.7 If red control or test lines are visible before the extraction fluid is added, the LFD might be damp or wet. Repeat the method with new LFD trays.

6.8 Interference by fuel system icing inhibitor (FSII), typically diethyleneglycol monoethyl ether, is not normally an issue, providing the concentration in the fuel or water phase does not exceed levels typically encountered in treated fuels.

6.8.1 Excessive FSII concentrations can delay wicking from the normal 10 min (see 10.1) interval to as long as 1 h.

6.8.2 When FSII is added to fuel at concentrations greater than those stipulated in most fuel specifications, or when very high concentrations of FSII partition to a sample water phase tested, or both occur, very high concentrations of FSII can be captured in the extraction fluid.

6.8.3 This test method is still usable when FSII interference is present, but if LFD control lines are not visible (see 10.11), LFD should be observed at 10 min intervals for up to 1 h.

6.8.4 If LFD control lines (see 10.11) are not visible within 1 h, the test results are not valid

6.9 Additives designed to retain water in suspension or diesel fuels containing high levels of FAME might delay rate of extraction fluid separation but will have no effect on the run time of the test or the results. See Note 4 in 10.2.

7. Reagents and Materials

7.1 *Antigen Extraction Fluid*[7]

7.2 *LFD Tray*—integrated assembly containing a tray of six paired LFDs for the detection of *H. resinae*, other fungi, and bacteria.[7]

NOTE 1—Each pair of LFDs includes one LFD to detect intermediate antigen concentrations (1.3) and one to detect antigen concentrations at the upper detection limit (1.3).

7.3 *Polyethylene Extraction Bottle,* HDPE, 175 mL with a dropper nozzle delivering 0.1 mL per drop.

7.4 *Disposable Pipet,* Pasteur, 5 mL.

NOTE 2—All of the above are contained within the FUELSTAT PLUS test kits.[7]

8. Hazards

8.1 Hazards are typical of those experienced when handling fuel. There are no additional hazards associated with this test method.

9. Sampling

9.1 Samples shall be collected and handled in accordance with Practice D7464.

9.2 A minimum sample of 150 mL of fuel, or fuel/aqueous mix, or 15 mL of fuel-associated aqueous solution alone is required to perform this test method.

NOTE 3—As part of the overall analysis of the fuel, it is recommended that a visual inspection is undertaken before this test method is carried out, using Test Method D4176.

10. Procedure

10.1 Collect sample in accordance with 9.1.

10.2 Shake sample for approximately 30 s and allow to settle for 12 min ± 1 min. Use separated water, fuel, or a mixture of both, as described in 10.6.

NOTE 4—The required settling time will depend on from where in the fuel phase the specimen for test is withdrawn after shaking. For example, a typical 200 mL sample allowed to settle for 12 min will theoretically have no suspended free water remaining in any fuel phase. However, a 1 L sample settled for the same time will still have suspended water in most of the fuel. Test results are likely to vary based on the location from within the partially phase-separated sample and the means by which the test specimen is retrieved.

10.3 The polyethylene sample extraction bottle has a flat transit cap, unscrew and remove this (see Fig. 1).

10.4 Remove LFD tray from foil pouch and label the LFD tray (Fig. 2) with specimen details and date of collection.

NOTE 5—LFD rows on the tray are labeled 'high' and 'low.' This is a prompt to remind which row should be read to indicate lower contamination levels and which for higher.

10.5 Visually inspect the sample to identify if water is present in accordance with the procedures described in Test Method D4176 (see 10.2).

10.6 *Extraction*—Select from extraction procedure A, B, or C, based on volume of visible water in sample:

10.6.1 *Extraction Procedure A*—If there is ≥15 mL water in the sample, transfer this water into the polyethylene sample extraction bottle (10.3) to the *water phase line*.

10.6.2 *Extraction Procedure B*—If there is visible water present, but is <15 mL, transfer all of the water into the polyethylene sample extraction bottle (10.3) and then transfer fuel from the sample up to the *fuel phase line*.

NOTE 6—Appendix X5 provides instructions for the use of a disposable syringe and an extraction tube to transfer the sample into the polyethylene sample extraction bottle (10.3). The disposable syringe and extraction tube or the disposable pipet (7.4) may be used for the sample transfer.

[7] The sole source of supply of the LFD Extraction Fluid and FUELSTAT PLUS (trademarked) test kits known to the committee at this time is Conidia Bioscience Ltd., Egham, TW20 9TY, UK. If you are aware of alternative suppliers please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

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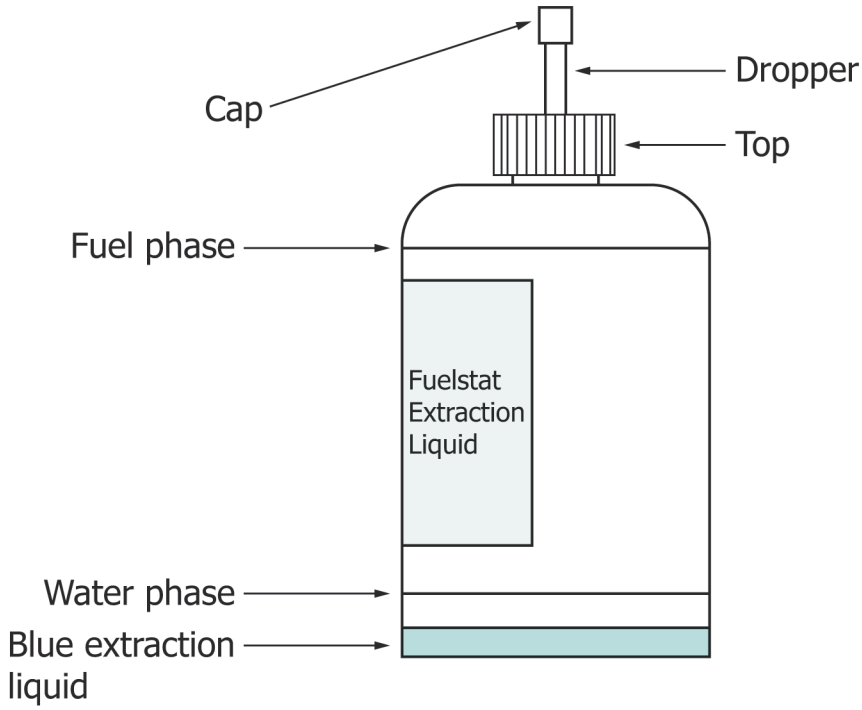

FIG. 1 Polyethylene Sample Extraction Bottle

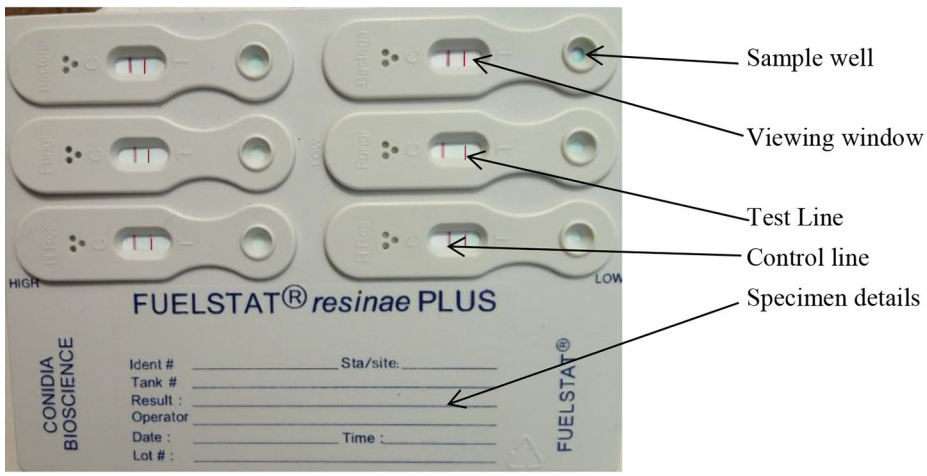

FIG. 2 LFD Tray

10.6.3 *Extraction Procedure C*—If there is no visible water present, dispense fuel into the polyethylene sample extraction bottle (10.3) up to the *fuel phase line*.

10.6.4 Secure the dropper top onto the bottle and shake the bottle vigorously for 5 s.

10.6.5 Invert the bottle and allow the extraction fluid or extraction fluid and water to settle into the dropper nozzle.

NOTE 7—The extraction fluid extracts antigen from the specimen. For specimens that contain fuel or fuel and aqueous phases, two liquid phases form in the cap of the inverted polyethylene extraction bottle: Extraction fluid containing any antigens from the fuel, and fuel itself. For specimens that include only aqueous solution, a homogeneous, blue-tinted solution forms a single phase.

NOTE 8—Aviation fuel and standard diesels will normally be extracted within 1 min. Diesel meeting EN590 (that is, up to 7 % FAME content) can take up to 2 min, diesels with higher FAME content will take longer (time increases up to 3 min at 30 %), and 100 % biodiesel typically can take 4 min for the extraction fluid to settle out.

10.6.6 Blue extraction fluid must remain at the bottom of the specimen in the dispenser cap to prevent fuel being introduced to the sample well. Ensure that the bottle remains inverted until the specimen has been dispensed onto all LFD sample wells.

10.7 Place LFD tray onto a horizontal, flat surface.

10.8 Squeeze the bottle and discard the first three drops of the fluid.

NOTE 9—This ensures that no fuel is trapped in the neck. Only the blue extraction fluid, NOT the fuel from the specimen, shall be placed in the sample well. Fuel will not wick along the LFD and rehydrate the reagents, and will thereby cause the test to fail.

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10.9 Squeeze the bottle; dispense four drops of the extraction fluid into each of the six LFD sample wells.

10.10 Let the LFD rest on the flat surface for 10 min in order to allow antigen solution to migrate past test and control lines.

10.11 After 10 min, confirm that all six control lines are visible.

10.11.1 These lines confirm that the liquid from the extracted specimen has wicked the whole length of the LFD and has come into contact with all reagents immobilized on the test.

10.11.2 If the control lines are not visible and the extraction fluid fails to flow on the LFD, this usually means that insufficient extraction fluid has been added to the test well; additional drops shall be added, one at a time, until flow is achieved.

10.11.3 The volume of the specimen is controlled by an absorbent pad within the LFD sample well, so once this is saturated the required specimen volume has been achieved.

10.11.4 If the control lines are still not visible, this can mean that fuel has inadvertently been added directly to the well or viewing window. Discard the LFD and repeat steps 10.1 – 10.11 using a new LFD.

10.12 Read all LFDs at 15 min ± 1 min. Lines are stable up to 30 min but it is not recommended to read results after this period except as noted in 6.8.3.

10.12.1 Observe and record presence or absence of test line in each LFD viewing window.

NOTE 10—Appendix X4 provides instructions for the use of a mobile device application and corresponding computer data platform for capturing and recording readings photographically. The precision statement provided in Section 13 does not apply to results obtained per Appendix X4.

11. Interpretation of Results

11.1 The presence or absence of test lines in the viewing windows on the LFD determines the results.

11.2 Interpret test results on the basis of lines visible in LFD pairs (*H. resinae*, other fungi, and bacteria; see 10.12).

11.3 The results will be one of the following:

11.3.1 Negligible—Antigen concentration ≤150 µg/L in the fuel phase or ≤33 µg/mL in the aqueous phase. Test lines are visible on both LFDs within the pair (Fig. 3).

11.3.2 Moderate—Antigen concentration >150 µg/L to ≤750 µg/L fuel, or >33 µg/mL to ≤166 µg/mL aqueous phase. Test lines are visible only on the high-range (left) LFD within the pair (Fig. 4).

11.3.3 Heavy—Antigen concentration >750 µg/L fuel or >166 µg/mL aqueous phase. No test lines, only control lines are visible within the pair (Fig. 5).

11.4 Some of the antigens detected by this test method can persist after treatment with a biocide.

11.4.1 The antigen concentrations begin to decrease immediately upon the death of biocide-treated microbes.

11.4.2 Cell-free antigen will normally disappear within 24 h after cell death.

NOTE 11—Antigenic metabolites become undetectable within fuel-treatment microbicide manufacturers' recommended soak (exposure) periods. Consequently, post-treatment tests should not be performed until after the soak period has lapsed.

12. Report

12.1 The test report shall contain at least the following information:

12.1.1 A reference to this test method.

12.1.2 The type and complete identification of the product tested.

12.1.3 The result of the test.

12.1.3.1 For each test microorganism or group of microorganisms—bacteria, other fungi, and *H. resinae*—report test results as negligible, moderate, or heavy, based on the pattern of visible lines (11.1 – 11.3).

12.1.4 The date the test was carried out.

12.1.5 The time and date when the specimen was taken.

12.1.6 The type of facility or equipment tested.

12.1.7 The location of the sampling point and the sampling method.

13. Precision and Bias

13.1 *Precision and Bias*—No information is presented about either the bias of Test Method D8070 for measuring microbial antigen concentration in fuels or fuel-associated water since the results are in the form of semi-quantitative, attribute data.

13.1.1 Attribute data precision includes:

13.1.1.1 *Within-operator consistency—Probability* of disagreement between two single classifications obtained within a short time in the same location on essentially the same material within a short time.

13.1.1.2 *Between-operator consistency—Probability* of disagreement between two single classifications obtained within a short time in the same location on the same material, where each classification is obtained by a different operator.

13.1.2 Attribute data *accuracy* is the % misclassification rate between operators versus correct classification.

FIG. 3 Bacteria LFD Detecting ≤150 µg Antigen/L Fuel Phase or ≤33 µg Antigen/mL Aqueous Phase: Both Test and Control Lines Visible

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