Designation: D 6530 – 00

An American National Standard

Standard Test Method for Total Active Biomass in Cooling Tower Waters (Kool Kount Assay; KKA)[1]

This standard is issued under the fixed designation D 6530; 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 covers the determination of viable active biomass in cooling tower water in the range from 10^2 to 10^8 cfu/mL (1). It is a semiquantitative test method.

1.2 This test method was used successfully with reagent water, physiologic saline, and cooling tower waters. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

1.3 *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 and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazard statements, see Section 9.

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

D 1192 Specification for Equipment for Sampling Water and Steam in Closed Conduits[2]

D 3370 Practices for Sampling Water from Closed Conduits[2]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *snapping cup*—container provided for holding the sample and snapping tip of the vial.

3.2.2 *vial*—sealed glass ampoule under vacuum containing reagents for the Kool Kount Test.

3.3 *Symbol*:

cfu/mL—colony forming units per millilitre

4. Summary of Test Method

4.1 This test method consists of adding a specific volume of water to nutrients and a color indicator contained in a glass vial. The contents of the vial are then mixed and incubated at 95°F (35 ± 3°C; that is, in a shirt pocket, incubator, or heat block). The color of the sample after addition into the vial containing the nutrients and color indicator is yellow. Viable active biomass in the sample replicates using the nutrients provided and reduces the color indicator. At a critical biomass concentration, sufficient quantities of the color indicator are reduced resulting in a visible change in the indicator from the original yellow sample color to orange. The time required for conversion of the oxidized indicator to the reduced indicator resulting in an orange color as directly correlated with the concentration of viable active biomass in the water sample tested. High concentrations of active biomass in the sample produce the positive orange color more rapidly than low concentrations of viable biomass.

5. Significance and Use

5.1 This test method is useful for rapid determination of viable active biomass concentrations in cooling tower waters. The efficiency of cooling towers is directly affected by the concentration of biomass in the cooling tower waters. As biomass concentrations increase, biofilm formation occurs resulting in a decrease in the efficiency of heat exchange in the tower. Current tests for monitoring the biomass concentration in cooling towers require at least 36 h for growth of the microorganisms on a solid agar surface for counting. Replication of microorganisms over the 36-h period before results are available creates an aqueous environment which is no longer represented by the data generated. Timely test results can assist in minimizing biocide addition to control biomass concentrations. Kool Kount provides data within hours to allow for more precise control of active biomass concentrations in the waters.

6. Interferences

6.1 Halogens interfere with this test method by inhibiting microbial growth resulting in lengthy incubation periods before a positive orange color is produced suggesting better water quality. Addition of thiosulfate eliminates this interference and allows for testing of waters previously treated with halogens (not immediately prior to testing).

6.2 Reducing agents (that is, beta mercaptoethanol) may interfere in this test method by reducing the color indicator chemically. Rapid color development upon filling of the vials

[1] This test method is under the jurisdiction of ASTM Committee D-19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] *Annual Book of ASTM Standards*, Vol 11.01.

suggests a chemical rather than a biological reaction. Waters containing reducing agents which react with the color indicator are not suitable for testing with Kool Kount.

6.3 Avoid prolonged exposure (greater than 30 min) of filled or unopened KKA vials to sunlight to avoid false positive reactions.

6.4 Testing must not take place within 24 h of biocide addition.

7. Apparatus

7.1 The schematic arrangement of the KKA test kit is shown in Fig. 1.

7.2 *(Parts) of the KKA Test K*—Vial A (test vial), vial under vacuum containing nutrient and reagent on glass rod; Vial B (control vial), vial under vacuum containing nutrient only (does not contain a glass rod); snapping cup; and plastic safety sleeve.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[3] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type III of Specification D 1193.

8.3 *Reagents*—Kool Kount Test Kit: Fischer Scientific, Cole Parmer, Calgon, Sodium thiosulfate: [$Na_2O_3S_2$]

9. Precautions

9.1 Precautions should be taken when snapping the glass tip from the glass vial containing the reagents. The tip must be submerged for the vial to completely fill as a result of the vacuum in the vial. A protective sleeve is provided with the kit to cover any rough glass edges on the neck of the vial during incubation.

10. Sampling

10.1 Collect the sample in accordance with Specification D 1192, and Practices D 3370 as applicable.

11. Procedure

11.1 Rinse snapping cup at least twice with water to be tested. Place at least 20 mL of the sample to be tested in the snapping cup. Add two drops of the thiosulfate solution provided. Mix and allow sample to sit quiescently in the snapping cup for 15 min.

11.2 Submerge the tip of glass Vial A containing reagents in the sample to be tested (in the snapping cup). Place the tip in one of the grooves in the bottom of the snapping cup. Carefully press the vial toward the opposite wall of the cup to snap the tip allowing the vial to fill as a result of the vacuum in the vial.

11.3 Submerge the tip of control glass Vial B (no glass rod) in the same sample. Place the tip in one of the grooves in the bottom of the snapping cup. Carefully press the vial toward the wall of the cup to snap the tip allowing the vial to fill.

11.4 Place a protective sleeve on the neck of each vial to cover the sharp edges. Carefully invert vials several times to completely mix the reagent powders with the water sample.

11.5 Prepare the label with the sample designation, sample pH, sample temperature, and the time at which test was initiated. Place the sample label on the appropriate vial and label the control vial. Incubate vials at approximately 95°F (35 ± 3°C; heat block, shirt pocket, incubator).

11.6 Examine Vials A and B after 10 to 15 min for development of pink to red color indicative of chemical reaction, not biological activity.

11.7 Examine sample Vial A for color change (yellow [negative] to orange [positive]) after 30 min of incubation by looking through the flat base of the vials comparing the test sample vial (A) with the control vial (B). Examine Vial A for color change at hourly intervals by looking through the flat base of the vial and comparing with the control Vial B. An incubation of 10.5 h, corresponding to 10^2 cfu/mL, is the limit of this test method for estimation of viable active biomass.

11.8 Note the time when the color change (yellow to orange) occurs.

11.9 Determine the total elapsed time from test initiation to positive color development [time completed − time initiated = total elapsed time; that is, 1:15(1315; end point) − 10:15 (1015; initiation) = 3 h].

12. Calculation

12.1 Elapsed time for positive color development is directly correlated with viable active biomass concentration in the sample tested. Total elapsed time is converted to biomass concentration in accordance with the table in Fig. 2. This biomass concentration represents the viable active biomass level present in the sample tested.

13. Report

13.1 Report the results including sample pH and elapsed time for positive color development. The elapsed time is converted into viable active biomass concentration in accordance with the table in Fig. 2.

14. Precision and Bias [4]

14.1 This test method was tested by three laboratories. Two operators in each of the three laboratories analyzed each of four samples (three cooling tower samples and one spiked control sample) in triplicate. One operator at a fourth laboratory also participated in this test method. The collaborative test data

[3] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[4] Supporting data for the precision and bias statements have been filed at ASTM Headquarters. Request RR:D-1168.

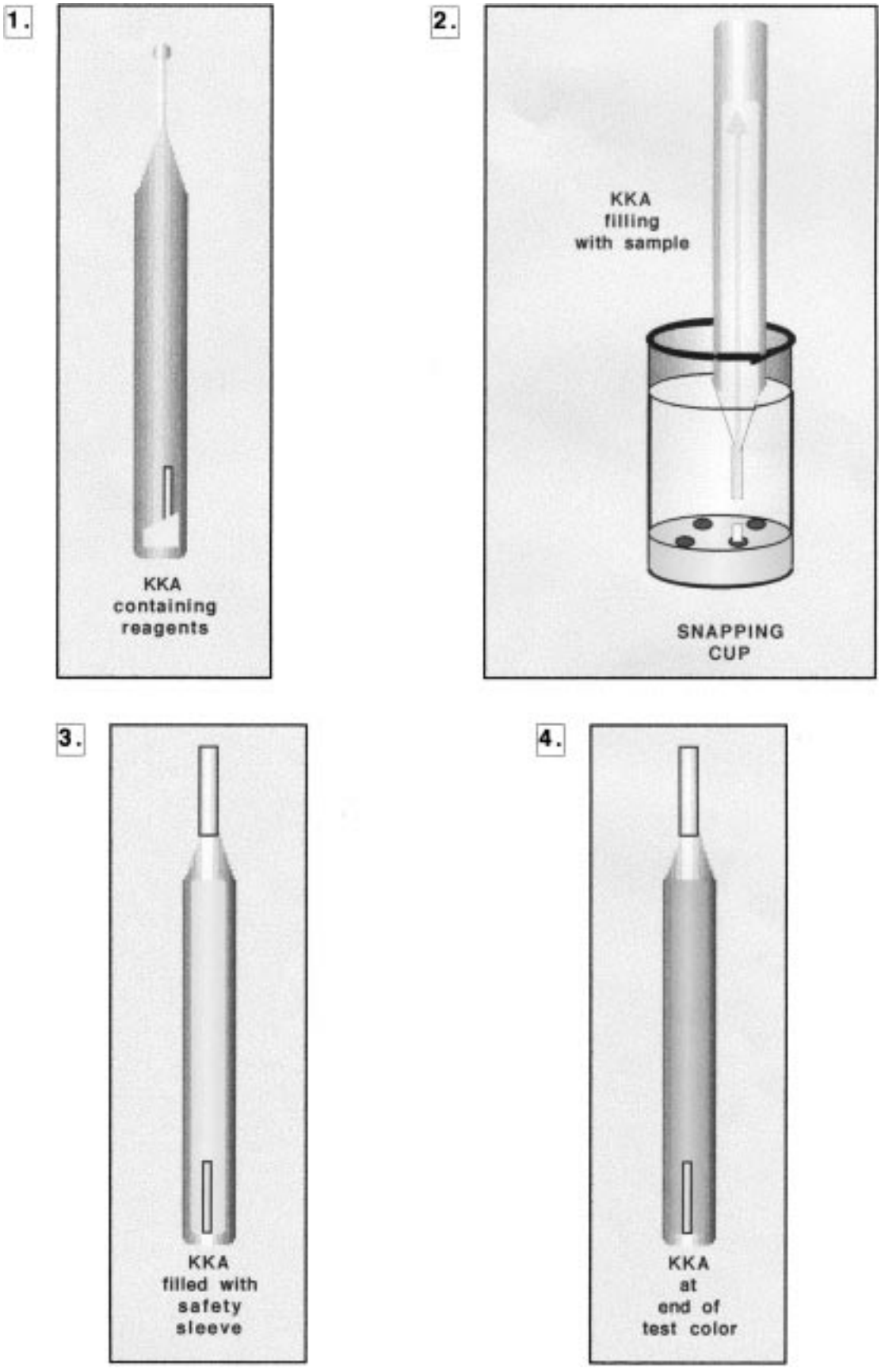

FIG. 1 Schematic of Kool Kount Test Kit

Time (Hours) to positive	cfu/mL (bacterial concentration)
0.5	$\geq 10^8$
2.5	10^7
4.0	10^6
5.5	10^5
7.0	10^4
9.0	10^3
10.5	10^2
12.5	10^1

FIG. 2 Standard Table for Determination of Viable Active Biomass in Cooling Tower Waters

were obtained using reagent water and cooling tower waters. For other matrices these data may not apply. See Table 1.

14.1.1 *Precision*—Allowances are made to precision and bias statement formats for test methods yielding a nonnumerical report of success or failure based on criteria specified in the procedure. No statement is made about either the precision or the bias of Method D-19.24(97-01), Kool Kount Test Method for measuring viable active biomass in cooling tower waters since the result merely states whether there is conformance to the criteria for success, that is, time to development of positive color, as specified in the procedure.

14.1.2 *Bias*—Comparison of KKA test results with results of the dip slide (current test method for cooling tower waters) and standard plate count methods did not show bias for higher or lower estimates if bioconcentration in the waters tested with Kool Kount. See Table 2.

14.2 Four independent laboratories (and a total of 7 operators) participated in the round-robin study. Under the allowances (exception to the precision and bias statement required by Practice D 2777 recommended by the results advisor) made in 1.3 of Practice D 277, these precision and bias data do meet existing requirements for interlaboratory studies of Committee D-19 test method.

15. Keywords

15.1 colorimetric bioassay; cooling tower water; triphenyl tetrazolium chloride; viable active biomass

TABLE 1 Round Robin Kool Kount Assay (KKA) Results

	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4
Sample No. 1[A]	9 h, 9 h, 9 h	6 h, 6 h, 6 h[A]	10.5 h, 10.5 h, 10.5 h	9 h, 9 h, 9 h
	9 h, 9 h, 9 h	7 h, 7 h, 7 h[A]	10.5 h, 10.5 h, 10.5 h	
Sample No. 2	>10.5 h, >10.5 h, >10.5 h[B]	>10.5 h, >10.5 h, >10.5 h	>10.5 h, >10.5 h, >10.5 h	>10.5 h, >10.5 h, >10.5 h
	10.5 h, 10.5 h, 10.5 h	0.5 h, 10.5 h, 10.5 h	10.5 h, 10.5 h, 10.5 h	
Sample No. 3	6 h, 6 h, 6 h	6 h, 6 h, 6 h	6 h, 6 h, 6 h	6 h, 6 h, 6 h
	7 h, 7 h, 7 h	7 h, 7 h, 7 h	7 h, 7 h, 7 h	
Sample No. 4	4 h, 4 h, 4 h	4 h, 4 h, 4 h	4 h, 4 h, 4 h	4 h, 4 h, 4 h
	4 h, 4 h, 4 h	4 h, 4 h, 4 h	4 h, 4 h, 4 h	

[A] Laboratories 2, 3, and 4 reported the presence of pink particulates in Sample No. 1 at 6–7 h. The aqueous phase did not turn a positive color until much later. Laboratory 2 did not record the time to development of positive color in the aqueous phase.
[B] >10.5 h = $<10^2$ cfu/mL, the lower detection limit of the method.

TABLE 2 Comparison of KKA, Dip Slide, and Standard Plate Counts

Sample	KKA	Dip Slide	Plate Count
No. 1	10^2– 8×10^2 cfu/mL[A]	1×10^3 cfu/mL	8×10^1 cfu/mL
No. 2	10^2– $<10^2$ cfu/mL	1×10^3 cfu/mL	5×10^2 cfu/mL
No. 3	2×10^4– 2×10^5 cfu/mL	1×10^5 cfu/mL	2×10^5 cfu/mL
No. 4	2×10^6 cfu/mL	1×10^7 cfu/mL	1×10^7 cfu/mL

[A] Does not include the results from Laboratory No. 2 representing the presence of pink particulates, not positive color development in the aqueous phase.