STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY (APA)

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This document is effective upon final approval

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1.0 Identification of Test Method

1.1 This method is the Standard Operating Procedure for the determination of Alkaline Phosphatase Activity (APA) in the laboratory of the South Florida Water Management District.

1.2 This method is based on "Determination of phosphate activity in lake water" by Petterson and Jansson.

2.0 Applicable Matrix or Matrices

2.1 This method can be applied to the determination of APA in surface waters collected within the South Florida Water Management District.

3.0 Detection Limit

3.1 The method detection limit for this method is determined using the procedure outlined in 40 CFR Part 136, Appendix B, using at a minimum seven replicates of a matrix sample. The value of sample is preferably in the range of three to five times the expected detection limit, but not more than 10 times the determined detection limit. The applicable method detection limit for this method is 1 nM/min

3.2 The MDL is reviewed annually by the QA officer and is subject to change. The current MDL is noted on the Test Control Parameters sheet and is available from the QA officer.

4.0 Scope and Application

4.1 The substrate used in this assay is methylumbelliferyl phosphate (MUP), which has low background fluorescence, thus allowing assay of wide variety of concentrations with very high sensitivity. MUP is prepared in a pH adjusted buffer and added into the sample. The phosphatase enzyme that may be present in the sample will hydrolyze MUP into methylumbelliferone (MU) and phosphate. MU fluoresces at a specific wavelength when excited with UV light, and can be quantified by a fluorometer. A computer-aided fluorescence Microplate Reader (Bio-tek Instruments, Inc.) is used to perform the analysis. The excitation wavelength is 360 nm and the emission wavelength is 460 nm.

4.2 After the MUP is added to the sample, the fluorescence is read at time zero and then re-read 30 minutes later. The fluorescence readings from MU in standards and samples are then used to calculate the enzyme activity in nM/min.

5.0 Summary of the Test Method

5.1 Phosphatases are enzymes that catalyze the hydrolysis of phosphomonoesters to orthophosphate and an alcohol: $R\text{-}PO_4 + H_2O <==> ROH + H_2PO_4$. These enzymes may be released into surface water as a result of disintegration of algal and bacterial cells. Phosphatase enzyme activity is inhibited by high dissolved organic carbon and inorganic phosphate contents.

5.2 Phosphatases are classified as either acid or alkaline, depending on the pH of the environment in which they exist. The determination of alkaline phosphatase activity (APA) is conducted in this method at pH 8.1 with a buffer solution (TRIS).

5.3 APA is sensitive to temperature. The sample is allowed to reach room temperature in the plate well using a shallow water bath. APA is measured at the controlled reading chamber/incubator temperature (26 ºC).

6.0 Definitions

6.1 A comprehensive listing of terms and definitions in common use in the SFWMD laboratory can be found in the Laboratory Quality Manual glossary. All acronyms are defined upon first use in this document. Only terms specific to this procedure are defined in this section.

7.0 Interferences

7.1 APA and fluorescence is sensitive to temperature. If the samples do not reach room temperature, the APA could be underestimated. If the temperature inside the instrument is uncontrolled and drifts to a high temperature due to the heat generation of the instrument, the APA could be overestimated.

7.2 If the substrate is not allowed to reach the room temperature, the method blank could be very negative and APA could be underestimated.

8.0 Safety

8.1 All personnel conducting this method should be familiar with the SFWMD Chemical Hygiene Plan and should have reviewed any pertinent Material Safety Data Sheets.

8.2 Wear safety glasses and a full-length, long-sleeved laboratory coat.

8.3 No hazardous wastes are generated by this procedure; liquid waste may be disposed of down the drain.

9.0 Equipment and Supplies

9.1 BioTek Multi-Mode Microplate Reader Synergy 2 attached to a computer with Gen5 v1.1 program.

9.2 Costar 48 well plate (3548).

9.3 Eppendorf pipette, 100 µL to 1000 µL adjustable pipette.

9.4 Eppendorf repipettor with 5 mL capacity tip.

9.5 Analytical balance, 0.1 mg sensitivity.

9.6 Volumetric flasks, 100 ml, 250 mL and 1000 mL capacity.

9.7 Class A volumetric pipettes, 5, 6, 8, 10, 15, 20 mL capacity.

9.8 Amber bottles (1L and 100 ml).

9.9 Freezer.

10.0 Reagents and Standards

10.1 All standards must be prepared monthly, using Class A glassware (pipettes and volumetric flasks). All standards (including S1 (laboratory reagent water)) and LCSs should be stored in **amber bottles**.

10.2 **Tris Stock Buffer (100 mM, pH 8.1 at 25 ^{o}C):** Into a 1 L class A volumetric flask containing 500 ml of Laboratory reagent water, dissolve one pack of Trizma pre-set crystals in foil pouches (Sigma Cat. # T-0944) or 13.98g Trizma pre-set crystals (Sigma Cat. # T8568) and 1.2037 g of magnesium sulfate ($MgSO_4$). Bring to volume with laboratory reagent water and mix. This solution is stable for six months.

10.3 **Tris Working Buffer:** Pour the Tris Stock Buffer (10.2) into a 10 L carboy and add 9 L of laboratory reagent water. This solution is stable for six months.

10.4 **Substrate:** Always keep Methylumbelliferyl phosphate ($C_{10}H_9O_6$), (MUP), (Sigma Cat.# M8883-5G) in the freezer. Weigh 0.128 g of MUP, quantitatively transfer into a 250 mL volumetric flask, and bring to volume with the TRIS stock buffer. Dispense into 15 mL plastic tubes and store in freezer. This solution is stable for six months. The old substrate solution should not be used and new substrate should be made when the background MU concentration in the MB is above 800 nM.

10.5 **Stock Standard (1000 µM MU):** Always keep 4 - Methylumbelliferone ($C_{10}H_8O_3$), (MU), (Sigma Cat.# M-1381) in a dessicator. Weigh 0.1762 g of MU, transfer into a 1L volumetric flask and dilute to volume with working Tris buffer. Transfer the solution into an amber bottle and store at room temperature.

10.6 **Solution A (50 µM MU):** Into a 100 mL volumetric flask, pipet 5 mL of stock standard MU solution and bring to volume with Tris working buffer.

10.7 **Working Standards Preparation**

10.7.1 Low Level Range Analysis.

Std's	Methylumbelliferone (MU) Conc.		Volume of Solution mL	Final Volume, mL (dilute with working TRIS buffer)
	µM	nM		
S1	0	0	Use laboratory reagent water	-
S2	0.1	100	10 ml S4	100
S3	0.4	400	10 ml S6	100
S4	1	1000	25 ml S6	100
S5	2.5	2,500	5 ml Soln A	100
S6	4	4,000	8 ml Soln A	100

10.7.2 High Level Range Analysis.

Std's	Methylumbelliferone (MU) Conc.		Volume of Solution mL	Final Volume, mL (use working TRIS buffer)
	µM	nM		
HS1	0	0	Use laboratory reagent water	-
HS2 (S4, Low)	1	1000	25 ml S6, Low	100
HS3 (S5, Low)	2.5	2,500	5 ml Soln A	100
HS5 (S6, Low)	4	4,000	8 ml Soln A	100
HS5	7.5	7,500	15 ml Soln A	100
HS6	10	10,000	20 ml Soln A	100

10.8 **LCS Stock (1000 µM MC):** Always keep 7-Hydroxy-4-methylcoumarin 97% ($C_{10}H_8O_3$), (MC), (Acros Cat. # 15638) in a dessicator. Weigh 0.1762 g of MC, transfer into a 1L volumetric flask and dilute to volume with working Tris buffer. Transfer the solution into an amber bottle and store at room temperature.

10.9 **LCS Solution A (50 µM MC):** Into a 100 mL volumetric flask, pipet 5 mL of stock standard MC solution and bring to volume with Tris working buffer.

10.10 **Working LCS Preparation**

LCS's	Methylcoumarin (MC) Conc		Volume of Solution mL	Final Volume, mL (use working TRIS buffer)
	µM	nM		
LCS1	0.3	300	10 ml LCS2	100
LCS2	3	3,000	6 ml Soln A	100
LCS3	5	5,000	10 ml Soln A	100
LCS4	7.5	7,500	15 ml Soln A	100

11.0 Sample Collection, Preservation, Shipment and Storage

11.1 Samples are collected using the procedures outlined in the SFWMD Field Sampling Manual or FDEP SOP's.

11.2 Samples for this method are collected, unfiltered, in a HDPE bottle and stored at ≤6 °C and not frozen. No additional preservation is required. Samples must be analyzed within 4 days.

11.3 Samples are transported to the lab in coolers containing wet ice or other devices to maintain the temperature of the sample at ≤6 °C and not frozen until delivery to the SFWMD laboratory.

11.4 Samples are stored in the laboratory in a cooler that is maintained at ≤6 °C and not frozen. Samples are removed from the cooler prior to testing and must be allowed to reach room temperature before addition of substrate.

12.0 Quality Control

12.1 Each analytical run should include working standards, blank, LCS's and a sample duplicate. LCS solutions are prepared using a different lot number and/or vendor than the stock used to make the Working Standard solutions.

12.2 The correlation coefficient of the standard curve must be ≥0.998.

12.3 A Duplicate analysis (sample selected at random) should be conducted for every 12 samples analyzed. The relative percent difference of the duplicate set is determined using the formula in section 15.3.

13.0 Calibration and Standardization.

13.1 All calibration standards should be prepared monthly.

13.2 Samples are analyzed on the Low Level calibration. If the fluoresce reading is over range, then the High Level calibration is used to analyze the over range samples.

14.0 Procedure

14.1 Create a batch for the analyses to be conducted. Refer to the SFWMD-LAB-SOP 5000 (current version). Download the Batch Worklist file from the LIMS and save the lab ID as a text file in the excel program.

14.2 Remove the samples from the refrigerator and allow them to reach room temperature. However, it will take very long time for 250-500 ml water samples to reach the room temperature. Alternatively, **let the samples warm to room temperature inside a shallow water bath for 15 minutes after transferring samples, standard and QCs to the assigned plate wells.**

14.3 Turn on the Fluorescence Plate Reader and allow to warm up for 30 minutes. The substrate (MUP) for the analytical run must also be defrosted, and allowed to reach room temperature. Make sure that everything in the substrate solution is dissolved after the defrosting.

14.4 Double click on Gen5 to open the program window.

14.5 Click "Experiment" to create a new experiment. Select the protocol "APAlow.prt" for the low concentration range APA sample or "APAhigh.prt" for the high range APA sample.

14.6 Click “plate 1” and then “sample IDs” at the left side of the toolbar menu. A New window will open. Click “Import” in the new window and select the lab ID text file to open. Click “OK” to close the new window.

14.7 Select “layout” from pull down icon (٧) and click the excel icon on the plate 1 data screen to export the well layout into the excel program. Print the well layout using the excel program.

14.8 Using the Eppendorf, pipette 1 mL standards and LCS’s. Pipette 0.9 mL of samples and method blank according to the well layout. Put the plate inside a shallow water bath for about 15 minutes to allow the samples to reach room temperature.

14.9 Click on the “Read Plate” icon on the top of the icon toolbar menu. The Plate reading/Runtime Prompts screen appears. In the analyst field put in the user name of the analyst. In the batch filed, enter the batch #.

14.10 Click “Read” and then save the experiment file as the batch #. The lamp will be warmed up.

14.11 Add 0.1mL (100 uL) of substrate MUP into each sample and MB well using a repipetter. **DO NOT ADD MUP SUBSTRATE TO STANDARD, OR LCS WELLS**. Immediately after adding the substrate, insert the plate into the instrument and click “OK”.

14.12 When the run is done, save the experiment file name as the batch # by clicking the save icon on the top icon toolbar menu**.** Check the correlation coefficient, MB, LCSs and duplicate result.

14.13 Verify that the sample fluorescence at time 0 and time 30 minutes is less than the fluorescence of 4000 nM standard. **Any sample with the fluorescence value > 4000 nM standard has to be analyzed on the High Range analysis protocol.**

14.14 For high range samples, follow procedure from Section 14.5 selecting the high range protocol.

14.15 Click the “Print” icon on the top icon toolbar menu to print the APA report.

14.16 All quality control data must be within the current established limits before printing the APA result report to the NuGenesis.

14.17 To transfer the files to LIMS, see SFWMD-LAB-SOP 5000 (current version).

14.18 Verify the correctness/acceptability of the results and note any discrepancies on the checklist.

14.19 Consult your supervisor before making any major changes, adjustments, and/or repairs to the instrumentation.

15.0 Calculations

15.1 To calculate final APA values in nM/min, the equation below is used:

$$APA(nM/min) = \frac{(MU\ conc.\ after\ 30\ min(nM) - MU\ conc.at\ time\ 0(nM)) \times 1\ ml}{30\ minutes \times 0.9\ mL}$$

15.2 The recovery of the LCS is calculated as follows:

$$\text{Recovery}(\%) = \frac{Observed\ Value}{True\ Value} \times 100$$

15.3 The relative percent difference (RPD) is calculated as follows:

$$RPD(\%) = \frac{|Value1 - Value2|}{(Value1 + Value2)/2} \times 100$$

16.0 Method Performance

16.1 This procedure was validated during method development to performance equivalent to the referenced method. Additional quality controls have been incorporated to assure consistent performance.

17.0 Pollution Prevention

17.1 The reagents and standards used in this method pose little threat to the environment when disposed down the sink with excess tap water.

17.2 Standards and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired standards and reagents to be disposed.

18.0 Data Assessment and Acceptance Criteria for Quality Control Measures

18.1 The sample holding time is 4 days. Notify the supervisor or team leader if any samples are out of the holding time.

18.2 LCS1, LCS2 (or LCS3 and LCS4 for high range samples), MB and a duplicate sample are analyzed with each analytical batch. The values must be within current quality control limits. If any of the quality control values fall outside the currently established

acceptance limits, follow section 24.2 for corrective actions for out-of control laboratory quality controls.

19.0 Corrective Actions for Out-of-Control Data.

19.1 See Section 24.2 for corrective actions for out-of control laboratory quality controls. The recommended corrective actions in Table 24.2 are intended as general guidelines to initiate the analytical trouble shooting process. In all cases, if the out-of-control condition persists beyond the second step in troubleshooting, notify your supervisor of the condition and request assistance.

20.0 Contingencies for Handling Out-of-Control or Unacceptable Data

20.1 If the out-of-control situation cannot be resolved by the corrective actions described in Section 19.0, and the instrument is functioning properly, the sample result(s) should be flagged with the appropriate qualifier code.

20.2 It may be necessary to obtain technical service to make repairs to instruments that are producing unacceptable data. See your supervisor for assistance in obtaining this service.

21.0 Waste Management

21.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

21.2 No hazardous wastes are generated by this method. All reagents and standards can be disposed in the sink with running water.

21.3 The samples collected for analysis by this method are not hazardous. They are disposed according to SFWMD-LAB-SOP-5510 (current version).

22.0 Instrument Maintenance

A maintenance logbook for each piece of instrumentation identified in this procedure shall be maintained by the analyst. The maintenance logbook must include the following items:

1. The name or laboratory designation of the instrument and the associated software;
2. The manufacturer’s name, type identification, serial number or other unique identification, and the SFWMD inventory control number;

3. Initial calibration records or other checks to confirm that the instrument complies with the method requirements;
4. The current location of the instrument;
5. Manufacturer's instructions, owner's manuals, or reference to their location;
6. Dates, results and copies of reports and certificates of all calibrations, adjustments, acceptance criteria, and the due date of the next calibration;
7. The maintenance plan as appropriate, and documentation of all maintenance carried out to date, including all routine and non-routine maintenance activities and reference material verifications;
8. Records of any damage, malfunction modification or repair to the instrument;
9. The date received and condition when first placed into service.

23.0 References

23.1 Pettersson, K. and M. Jansson. 1978. Determination of phosphatase activity in lake water- a study of methods. Verh. Internat. Verein. Limnol. 20:1226-1230.

23.2 Prof. Robert G. Wetzel. 1994. Personal Communication. Department of Biological Sciences, The University of Alabama, Tuscaloosa, Alabama 35487-0344, USA.

23.3 Bio-Tek Instruments, Inc. Synergy 2 Mulyi-Mode Microplate reader Operator's Manual.

23.4 Bio-Tek Instruments, Inc. Gent Getting Started Guide Microplate Data Collection & Analysis Software.

23.5 SFWMD-LAB-SOP 5000, current version.

23.6 SFWMD Laboratory Quality Manual, current version.

23.7 SFWMD (2010) APA method development/validation and sample holding time study.

24.0 Tables, Diagrams, Flowcharts and Validation Data.

24.1 Tables

TABLE 1.0 Well layout for the low range sample (48-well plate)

(A1) **S1**	(A2) **S2**	(A3) **S3**	(A4) **S4**	(A5) **S5**	(A6) **S6**	(A7) **LCS1**	(A8) **LCS2**
(B1) **S1**	(B2) **S2**	(B3) **S3**	(B4) **S4**	(B5) **S5**	(B6) **S6**	(B7) **LCS1**	(B8) **LCS2**
(C1) **MB**	(C2) **Sample 1**	(C3) **Sample 2**	(C4) **Sample 3**	(C5) **Sample 4**	(C6) **Sample 5**	(C7) **Sample 6**	(C8) **Sample 7**
(D1) **MB**	(D2) **Sample 1**	(D3) **Sample 2**	(D4) **Sample 3**	(D5) **Sample 4**	(D6) **Sample 5**	(D7) **Sample 6**	(D8) **Sample 7**
(E1) **Sample 8**	(E2) **Sample 9**	(E3) **Sample 10**	(E4) **Sample 11**	(E5) **Sample 12**	(E6) **Sample Duplicate**	(E7) **MB**	(E8) **LCS2**
(F1) **Sample 8**	(F2) **Sample 9**	(F3) **Sample 10**	(F4) **Sample 11**	(F5) **Sample 12**	(F6) **Sample Duplicate**	(F7) **MB**	(F8) **LCS2**

Table 2.0 Well layout for the high range sample (48-well plate)

(A1) **HS1**	(A2) **HS2 (S4, low)**	(A3) **HS3 (S5, low)**	(A4) **HS4 (S6, low)**	(A5) **HS5**	(A6) **HS6**	(A7) **LCS3**	(A8) **LCS4**
(B1) **HS1**	(B2) **HS2 (S4, low)**	(B3) **S3 (S5, low)**	(B4) **S4 (S6, low)**	(B5) **HS5**	(B6) **HS6**	(B7) **LCS3**	(B8) **LCS4**
(C1) **MB**	(C2) **Sample 1**	(C3) **Sample 2**	(C4) **Sample 3**	(C5) **Sample 4**	(C6) **Sample 5**	(C7) **Sample 6**	(C8) **Sample 7**
(D1) **MB**	(D2) **Sample 1**	(D3) **Sample 2**	(D4) **Sample 3**	(D5) **Sample 4**	(D6) **Sample 5**	(D7) **Sample 6**	(D8) **Sample 7**
(E1) **Sample 8**	(E2) **Sample 9**	(E3) **Sample 10**	(E4) **Sample 11**	(E5) **Sample 12**	(E6) **Sample Duplicate**	(E7) **MB**	(E8) **LCS4**
(F1) **Sample 8**	(F2) **Sample 9**	(F3) **Sample 10**	(F4) **Sample 11**	(F5) **Sample 12**	(F6) **Sample Duplicate**	(F7) **MB**	(F8) **LCS4**

24.2 Corrective actions for out-of-control laboratory quality controls.

LCS Activity	Acceptance Criteria	Recommended Corrective Action
Initial Instrument Blank Method Reagent Blank	<MDL response & value	Trouble shoot the instrument and determine cause of error (reagents, calibration standards, environment, equipment failure, etc), correct the problem, then reanalyze.
Initial Calibration Standards	Correlation coefficient ≥0.998	Re-analyze standards. If same response is obtained, trouble shoot the instrument & re-start analysis. If same response is obtained, prepare new standards & re-start analysis.
Quality Control or Check Standards	Accuracy within established limits. See Quality Manual	Re-analyze LCS check standard. If same response is obtained, trouble shoot the instrument & re-analyze. If same response is obtained, prepare new LCS & re-start analysis.
Replicate/Duplicate Sample	Precision within established limits. See Quality Manual	Re-analyze sample. If same response is obtained, trouble shoot the instrument & re-start analysis. If same response is obtained, prepare new duplicate & re-start analysis.

Note: The recommended corrective actions in Table 24.2 are intended as general guidelines to initiate the analytical trouble shooting process. In all cases, if the out-of-control condition persists beyond the second step in troubleshooting, notify your supervisor of the condition and request assistance.

24.3 Corrective actions for out-of-control field quality control samples.

Indicator	Acceptance Criteria	Recommended Corrective Action
Equipment Blank(EB) Trip Blank(TB)	<MDL or less than established limits	Laboratory should re-analyze positive field blanks to confirm results.
Field Duplicates (FD) Replicate Samples (RS) Split Samples (SS)	Precision within limits See Quality Manual (only if values >PQL)	Laboratory should re-analyze failing field QC samples to confirm results.

24.4 Instrument parameters setting in the Gen5 software

Tab	Item	Setting
Reading Procedure	Plate Type	Costar 48 well
	Set Temperature	Temperature: Setpoint 26 ºC
	Read	Read: (F) 360/40, 460/40
	Delay	Delay for 0:30:00
	Read	Read: (F) 360/40, 460/40
Filter/Probe Sets	Excitation	360/40
	Emission	460/40
	Optics Position	Top 400 nm
	Sensitivity	50 (low range) 45 (high range)
	Top Probe Vertical Offset	7.00 nm
Instrument Sets	Step Label	<default>
	Detection Method	Fluorescence
	Read Type	Endpoint
	Read Speed	Normal
	Light Source	Tungsten

CHECKLIST

Parameter / SOP #

Analysis Date

HBN #

Analyst Review	ANALYST YES	ANALYST NO	SPVR YES	SPVR NO
Initial Calibration and Quality Control criteria met				
Continuing Quality Control and Calibration criteria met				
Sample Analysis				
Sample holding times met				
Reported Data readings within the calibration range				
Samples analyzed out of designated bottle set (if not note in comments)				
Other				
Required forms completed and submitted				
Above cited SOP read and followed				
All reagents and standards documented as required				
Electronic Content				
Post Run Annotations				
All required documents printed / scanned into LIMS				

Comments: _____

Analyst: _____ **Date:** _____

Supervisor: _____ **Date:** _____

QA: _____ **Date:** _____

25.0 SOP Addendums and Changes

26.0 Document History

Document Version	Date Issued	Description	Author
3160-003	11/12/09	New revision of SOP	C. Janson SFWMD
3160-004	8/12/10	New revision of SOP	M. Zhou SFWMD
3160-005	5/10/13	Updated SOP	M. Zhou SFWMD