

FLEX-REAGENT™

ACETIC ACID

Enzymatic, UV-Method

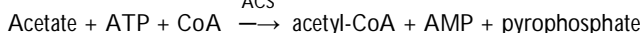
Product #: AA-F60 (30 Tests), AA-F150 (75 Tests)
AA-F500 (250 Tests)

INTENDED USE

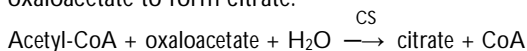
Acetic Acid FLEX-REAGENT™ is intended for determination of acetic acid (acetate) in wine, foodstuffs and other liquid samples.

METHODOLOGY & CHEMICAL PRINCIPLES

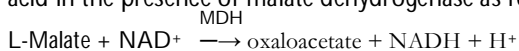
The enzyme acetyl-CoA synthetase (ACS) catalyzes the reaction of acetic acid (acetate) with coenzyme A (CoA) in the presence of adenosine-5'-triphosphate (ATP), producing acetyl-CoA.¹



Citrate synthase (CS) catalyzes the reaction of acetyl-CoA and oxaloacetate to form citrate.



Oxaloacetate, consumed in this reaction, is formed from L-Malic acid in the presence of malate dehydrogenase as follows:



The increase in NADH concentration is measured at 340nm and is the basis for calculation of acetic acid concentration in the sample.

REAGENTS

Acetic Acid FLEX-Reagent Concentration	Quantity/Kit	as Formulated		
		30T	75T	250T
1. Opti-Buffer Solution				
Malic Acid	4.19 G/L	20mL	50mL	170mL
MgCl ₂	2.09 G/L			
Preservatives, stabilizers	Buffered at pH 8.4			
2. Co-Enzyme Powder				
ATP	25 G/L	4mL	10mL	35mL
Co-Enzyme A	2.57 G/L			
NAD	12.3 G/L			
3. MDH/CS Solution				
L-Malate Dehydrogenase	458 U/mL	1.3mL	3.3mL	11mL
Citrate Synthetase	2.1 U/mL			
4.a) ACS Enzyme Powder				
reconstitute to:		1.3mL	3.3mL	11mL
Acetyl Co-A Synthetase	15.4 U/mL			
b) ACS Diluent				
		1.5mL	4mL	12mL
5. Acetic Acid Standard				
0.15 G/L		1mL	1mL	5mL

A 5-Level Acetic Acid Standards Kit is available from Unitech

REAGENT PREPARATION & STORAGE

Components as supplied are stable through the labeled expiration date when stored at 2-8°C. TEA Buffer, MDH/CS Solution, and Acetic Acid Standard are ready to use.

Reagent Preparation: Dissolve the Co-Enzyme Powder (vial #2) with the volume of deionized water indicated on the label.

Dissolve the ACS Enzyme Powder by adding the volume of ACS Diluent (vial 4.b stated on the ACS Enzyme (vial 4.a) label.

Gently mix by inversion to assure dissolution. The Co-Enzyme Solution (vial #2) and ACS Enzyme Solution (vial #4) are stable for 3 months when stored at 2-8°C. Write solution expiration dates on these labels.

Working Reagent: Prepare sufficient WRgt for all samples and standards in the assay using clean glassware, according to the examples in (either Automated or Manual) table below.

ChemWell Optimized Protocol (automation)

- a. Prepare **WORKING REAGENT** according to the examples in the tables below. Discard turbid working reagent or if the 340nm absorbance is greater than 0.15.

AUTOMATED TESTING WRgt is stable for 5-days refrigerated.

	25 Tests	55 Tests	100 Tests
Opti-Buffer (#1)	3.00 mL	5.0 mL	8.0 mL
MDH/CS Solution (#3)	0.200 mL	0.320mL	0.520mL
Deionized Water	6.00 mL	10.0 mL	16.0 mL
WRgt (Approx.Total)	9.2 mL	15.3 mL	24.5 mL

(# of Tests accounts for Reagent Bottle dead volume)

Solutions b. & c. below will be placed in reagent rack.

- b. Co-Enzyme Solution (Stable 3-months as solution.)

- c. **DILUTED ACS** ACS Enzyme Solution #4' is dilute 6-fold in DI Water. Prepare daily; ACS-Diluted is stable for 24hr 5°C.:

	25 Tests	55 Tests	100 Tests
DI Water	0.6	1.2	2.1
ACS Enz Soln. #4	0.12	0.24	0.42
Diluted ACS (Approx.Total)	0.7	1.42	2.5

MANUAL TESTING WRgt is stable 1-day refrigerated

	1 Test	8 Tests	13 Tests	24 Tests
Opti-Buffer (#1)	1 mL	5 mL	8 mL	15 mL
Co-Enzyme Soln. (#2)	0.2mL	1.0mL	1.6mL	3 mL
Deionized Water	2 mL	10 mL	16 mL	30 mL
WRgt (Approx.Total)	3 mL	16 mL	26 mL	49 mL

Pipette into Cuvettes	Reagent Blank Cuvette	Reaction Cuvettes
Sample		20µL
DI water	20µL	
Working Reagent	2 mL	2 mL
Mix and read absorbances (A ₀). Add Solution #3.		
MDH/CS Solution #3	40 uL (1 drop)	40 uL (1 drop)
Mix, wait 3 minutes, and read absorbances (A ₁).		
ACS Solution #4	40 uL (1 drop)	40 uL (1 drop)
Mix, wait 15+ minutes, and read absorbances (A ₂).		

System parameters: Wavelength 340 nm, Absorbance Range 0-2A, pathlength 1.0 cm. Schematically, the assay protocol is:



PROCEDURE

1. Allow Working Reagent (WRgt) to reach room temp.
2. Pipet water into the Reagent Blank cuvette and pipet standards and samples into cuvettes as shown.
3. Zero spectrophotometer with DI water. Read initial absorbance (A_0) values for Rgt Blank, Std, and Samples.
4. Add Solution #3, wait 3 minutes and read absorbance (A_1). Add Solution #4, wait 20 - 30 minutes; read absorbance (A_2). If free acetic acid is to be determined separately from acetate esters, read additional A_2 values at 35 and 40 minutes.

ΔA_{AcAc} & G/L CALCULATIONS

Acetic acid concentration is not directly proportional to ABS $A_2 - A_0$ due to the equilibrium of the MDH mediated reaction¹.

Calculate ΔA_{AcAc} values as follows: $\Delta A_{AcAc} =$

$$\left[\frac{(A_2 - A_0)_{\text{sample}} - \frac{(A_1 - A_0)_{\text{sample}}^2}{(A_2 - A_0)_{\text{sample}}}}{(A_2 - A_0)_{\text{sample}}} \right] - \left[\frac{(A_2 - A_0)_{\text{blank}} - \frac{(A_1 - A_0)_{\text{blank}}^2}{(A_2 - A_0)_{\text{blank}}}}{(A_2 - A_0)_{\text{blank}}} \right]$$

If desired, a correction permits determination of free acetate, excluding that released by hydrolysis of acetate esters. Refer to "SAMPLES" section "Correction for Esters" below.

1. Extinction Coefficient

- a. "Flex Calculator™ Method": Open the "Flex Calculator™ Acetic Acid" file in Excel™* or comparable spreadsheet and enter the Absorbance values (A_0 , A_1 , and A_2) for the Blank and each Sample cuvette. The ΔA_{AcAc} and G/L values will be calculated automatically.

* Excel is a Trademark of Microsoft

b. Manual Calculation Method

Calculate ΔA_{AcAc} values as described above.

$$G/L = \frac{MW \times T.V. \times d.f.}{(\epsilon)(P)(1000\text{mg/g})(SV)} \times \Delta A_{Acetic\ Acid} = 1.001 \Delta A_{AcAc}$$

Where:

MW = 60.05 G/mole

TV = total reaction vol. (2 + 0.02 + 0.04 + 0.04) = 2.10 mL

SV = sample volume (0.020 mL)

ϵ (absorptivity of NADP) = 6.3 @ 334-340nm [or 3.4 @ 365nm]

P = 1 cm light path

d.f. = dilution factor (e.g. "5" for samples diluted 1:5)

Adjust calculations if alternate WRgt & SV are used. Inaccuracy in sample volume delivery will affect results with this calculation method; use calibrated micropipettes.

2. Single-Point Standard Method, eg 0.15G/L Acetic Acid Std

Calculate ΔA_{AcAc} values as described above.

$$\text{Acetic Acid G/L} = 0.15 \times \frac{\Delta A_{AcAc}(\text{sample})}{\Delta A_{AcAc}(\text{standard})}$$

G/L CALCULATIONS (simplified for Automation)

Multi-Point Standard Method - Run a 5-point standard curve.

Calculate $\Delta A = A_2 - A_1$ for Blank, Standards, and Samples.

Calculate Delta Values = $\Delta A - \Delta A_{\text{Blank}}$ for Stds and Samples.

Plot Delta Values vs. concentration for each standard.

Calculate G/L of each sample from this curve.

SAMPLES

Significance of Measurements: Acetic acid is the primary acid formed during wine spoilage; legal limits vary by wine region. Volatile acidity determined by distillation consists primarily of acetic acid, propionic and lactic acids.² The uv-enzymatic method for acetic acid specifically measures acetic acid.

Correction for Esters: Acetic acid esters (e.g. ethyl acetate) frequently are found in the presence of acetic acid. Ethyl acetate is slowly hydrolyzed under assay conditions and is responsible for creep reactions. The effect of acetic acid esters may be eliminated in the calculations as follows: the value of A_2 at the time of ACS Enzyme addition may be extrapolated from the A_2 slope between 15 - 20 minutes; this corrected A_2 will provide the acetic acid concentration. To estimate total acetate content, allow reactions to reach endpoint (until Abs values stabilize) and compute as per "Calculations", above.

Linearity: The linear range of this assay for white/rosé wine is 0.07 - 0.75 G/L, and for red wine 0.07 - 0.45 G/L. Dilute over-range samples with deionized water and reassay. When $\Delta A_{AcAc} \leq 0.025$, repeat analysis with a 'neat' (or less dilute) sample, or with a larger sample volume.

Clarification: Turbid samples should be filtered. Fermentation samples may be clarified by centrifugation (if necessary), covered, and placed into a water bath at 80°C to inactivate fermentation enzymes. Pigments in the sample may interfere with enzymatic determinations, typically only when SV larger than 100 μL are used. If an unacceptably high sample blank absorbance is obtained, mix 10 mL juice and approximately 0.1G polyamide powder or polyvinylpoly-pyrrolidone (PVPP), stir for 1 minute and filter.

QUALITY CONTROL

An Acetic Acid standard should be included in each assay; the standard provided may be used in calculations, or in monitoring reaction completion. For quality control, calculate the "% Expected" for the standard with an established acetic acid value. Factors that may affect the performance of this test include proper instrument function, temperature standard, glassware cleanliness, and pipetting accuracy.

NOTES:

1. Wavelength: NADH absorbance maximum is 340nm; 334-340nm determinations provide the best analytical discrimination. While less sensitive, 365nm provides a broader measuring range.
2. Suggested Dilutions for red wine samples:

Estimated Acetic Acid	Dilution
0.45 to 2.25 G/L	1:5
≥ 2.25 G/L	1:10
3. Note that high alcohol content slows the reaction; permit 20 - 25 minute reaction time for A_2 these samples.

REFERENCES

1. Methods of Enzymatic Analysis, Bergmeyer, H.U., 2nd Edition, Vol 1, 112-117, Academic Press, Inc. New York.
2. Chemistry of Winemaking, Advances in Chemistry Series 137. A. Dinsmoor Webb. American Chemical Society, 136-137, (1974.)
3. Green, A, in "Biochemistry of fruits and their Products," Vol 2, Ch11, AC Hulme, ed., Academic, London & New York, 1971.
4. Amerine, MA, Thoukis, G. Vitis (1958) 1, 224.

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