SIGMA DIAGNOSTICS®

ALCOHOL (ETHANOL)

(Procedure No. 332-UV)

INTENDED USE

Sigma Diagnostics Alcohol reagents are for the quantitative, enzymatic determination of alcohol in serum, plasma, whole blood or urine at 340 nm.

SUMMARY

Ethanol is a test frequently performed in medicolegal cases to determine if an individual is intoxicated. Bucher and Redetzki[1] devised a convenient and accurate enzymatic method for measuring ethanol. The Sigma Diagnostics alcohol procedure is a modification of that method.

PRINCIPLE

$$\text{Ethanol} + \text{NAD} \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH}$$

Alcohol dehydrogenase (ADH) catalyzes the oxidation of alcohol to acetaldehyde with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH.[2] The consequent increase in absorbance at 340 nm is directly proportional to alcohol concentration in the sample.

REAGENTS PROVIDED

NAD-ADH SINGLE ASSAY VIAL, Catalog No. 330-1
NAD, 1.8 µmol, ADH (yeast), 150 units, and buffer salts.

NAD-ADH MULTI-ASSAY VIAL, Catalog No. 332-5
NAD, 9.6 µmol, ADH (yeast), 800 units, buffer salts and stabilizers.

GLYCINE BUFFER REAGENT, Catalog No. 332-9
Glycine, 0.5 mol/L, pH 9.0, with trapping agent.

ETHANOL STANDARD SOLUTION, Catalog No. 330-20
Ethanol, 0.08% (w/v).

TRICHLOROACETIC ACID SOLUTION, Catalog No. 331-7
Trichloroacetic acid, 6.25% (w/v). (Required for whole blood or where it is desired to deproteinize highly colored or turbid samples.)

PRECAUTIONS:

Alcohol reagents are for "In Vitro Diagnostic Use". Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state and federal laws.

All reagents should be closed immediately after use to avoid contamination by alcohol or other vapors. Do not use volatile solvents in the work area when performing assays because of possible contamination of specimens or reagents with vapors.

Glycine Buffer Reagent is TOXIC and MAY CAUSE CANCER. May cause heritable genetic damage. May cause sensitization by inhalation or skin contact. Do not breathe vapor. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves and eye/face protection. Target organ(s): kidneys and liver.

Trichloroacetic Acid Solution CAUSES BURNS. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Take off immediately all contaminated clothing. Wear suitable protective clothing, gloves and eye/face protection. Do not breathe vapor. Vesicant. Target organ(s): Central nervous system.

Refer to Material Safety Data Sheets for any updated risk, hazard or safety information.

PREPARATION:

NAD-ADH Reagent is prepared by reconstituting contents of NAD-ADH vials with volume of Glycine Buffer, Catalog No. 332-9, indicated below. Stopper vials and immediately mix several times by inversion. DO NOT SHAKE. Allow reagent to reach room temperature before use.

NOTE: If reagent is to be used with a discrete analyzer, please refer to the appropriate Sigma Diagnostics application procedure for reagent preparation.

NAD-ADH Catalog No.	Method	Volume of Glycine Buffer Required
330-1 (Single assay)	Procedure 1 (serum, Plasma or urine)	3.0 mL
330-1 (Single assay)	Procedure 2 (whole blood)	2.9 mL
332-5 (Multi-assay)	Procedure 1 or 2	16 mL
332-30* (Multi-assay)	Procedure 1 or 2	100 mL

* NAD-ADH, Catalog No. 332-30, is offered as an individual reagent for customers with high reagent volume needs.

STORAGE AND STABILITY:

Store NAD-ADH Single Assay Vials below 0°C in desiccator package provided. Allow package to warm to room temperature before removing vials as needed. Quickly reclose package and place back in freezer. Store NAD-ADH Multi-Assay Vials below 0°C. Store other reagents in refrigerator (2–8°C). Reagents are stable until expiration date on label.

NAD-ADH Reagent is stable for 8 hours at room temperature (18–26°C), 3 days in refrigerator (2–8°C) and for at least 2 months frozen.

DETERIORATION:

NAD-ADH Reagent absorbance, when measured in a 1-cm lightpath at 340 nm vs water as reference, is normally less than 0.2. However, the reagent is suitable for use at an absorbance up to 0.3.

OPTIONAL REAGENTS

ETHANOL CONTROL-L, Catalog No. E 5133
Contains approximately 0.07% ethanol (w/v) in human serum base.

ETHANOL CONTROL-H, Catalog No. E 5258
Contains approximately 0.2% ethanol (w/v) in human serum base.

ETHANOL STANDARDS SET, Catalog No. 332-11
Set contains aqueous standards with ethanol concentrations of 0.05, 0.1 and 0.3% (w/v).

DISCRETE ANALYZER APPLICATIONS

Application procedures using Sigma Diagnostics Alcohol (Ethanol) reagents are available for various automated instruments. Please contact Sigma Diagnostics Technical Services Department (1-800-325-0250 or 314-771-3122 collect) for more information.

SPECIMEN COLLECTION AND PREPARATION

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-T2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.

Ethanol should not be used to sterilize equipment or in the swab used at the site of venipuncture. Serum, plasma, urine or whole blood are suitable as samples. Anticoagulants which may be used[3] for collecting whole blood include potassium oxalate, sodium fluoride, sodium citrate, EDTA and heparin. Specimens that are well stoppered and refrigerated may not show appreciable loss of ethanol for several days.[4]

INTERFERING SUBSTANCES:

Certain higher aliphatic alcohols will also react in the described procedure, but more slowly than ethanol. A comparison of relative interference by various alcohols is given below.[5]

Substance	Approximate Reactivity (%) Determined by Sigma Procedure No. 332-UV*
Ethanol	100
n-Butanol	40
Isopropanol	8
Methanol	0
Ethylene glycol	1
Acetone	0

*NOTE: Relative reactivity will depend upon the time at which the reaction absorbance is measured. Increased interference may be noted with increased reaction times.

Certain drugs and other substances are known to influence circulating ethanol levels.[6]

MANUAL PROCEDURE

MATERIALS PROVIDED:

Reagents - See "Reagents" section.

MATERIALS REQUIRED BUT NOT PROVIDED:

Instrument: Any instrument that will transmit light at a wavelength of 340 nm is suitable for this procedure. If a narrow-bandwidth spectrophotometer is used, the change in absorbance can be employed directly for calculating the final results. Wide-band spectrophotometers (e.g., Coleman Jr. II, Model 6/20 or B&L Spectronic 20) require a calibration curve for deriving final results. Details are provided in the "Calibration" section.

MATERIALS:

Cuvets with optical properties suitable for use at 340 nm
Pipeting devices for the accurate delivery of volumes required for the assay
Timer
Centrifuge and stoppered centrifuge tubes are needed for whole blood

PROCEDURES

PROCEDURE NOTES:

1. Samples which are hemolyzed, moderately turbid or icteric, may be assayed directly in the procedure for serum, plasma or urine since their contribution to apparent alcohol concentration is less than 0.01% (10 mg/dL). Samples which are markedly turbid or markedly icteric should be assayed using the procedure for whole blood which deproteinizes the sample.
2. A new BLANK must be prepared for each set of TESTS. Do not use a BLANK from a previous test series.
3. Since urine may contain unknown substances which absorb at 340 nm, it may be appropriate to include a urine sample blank, by adding 10 µL urine to 3.0 mL Glycine Buffer Reagent. The absorbance value is then subtracted from that of the TEST prior to calculating the results.
4. It has been suggested that alcohol concentrations less than 10 mg/dL (0.01%) not be reported numerically, but designated as negative for alcohol.[7]

PROCEDURE 1 (SERUM, PLASMA OR URINE):

1. **Using NAD-ADH Single Assay Vials**
   a. Label NAD-ADH Single Assay Vials, Catalog No. 330-1, for BLANK and TESTS.
   b. To each vial, add 3.0 mL Glycine Buffer Reagent, Catalog No. 332-9.
   c. Cap and invert gently several times to dissolve contents. DO NOT SHAKE.

   Using Multi-Assay Vial
   a. To NAD-ADH Multi-Assay Vial, Catalog No. 332-5, add 16.0 mL Glycine Buffer Reagent, Catalog No. 332-9.
   b. Cap and invert gently several times to dissolve contents. DO NOT SHAKE.
   c. Label test tubes for BLANK and TESTS, and add 3.0 mL NAD-ADH Solution prepared above.
2. To BLANK add 0.01 mL (10 µL) deionized water.
   To TEST(S) add 0.01 mL (10 µL) sample.
   Cap or cover immediately and mix by gentle inversion.
3. Allow solutions to incubate for 10 minutes at any temperature between 22°C and 37°C.
4. Transfer solutions to cuvets and measure absorbance of TESTS at 340 nm vs BLANK. Complete readings within 10 minutes.
   To determine alcohol concentration, refer to "Calculations" section.

PROCEDURE 2
(WHOLE BLOOD, MARKEDLY TURBID OR MARKEDLY ICTERIC SAMPLES):

Sample Deproteinization

Prepare protein-free supernatants as follows:

1. Pipet 1.8 mL Trichloroacetic Acid Solution, Catalog No. 331-7, into a centrifuge tube.
2. While swirling tube, slowly add 0.2 mL sample.
3. Stopper tube immediately and mix by vortexing. Allow to stand at room temperature for approximately 5 minutes.
   NOTE: If clumping of blood occurs, disperse clumps by crushing with a glass rod and remix.
4. Centrifuge at approximately 2000 rpm for 5 minutes to obtain a clear supernatant.

Assay

1. **Using NAD-ADH Single Assay Vials**
   a. Label NAD-ADH Single Assay Vials, Catalog No. 330-1, for BLANK and TESTS.
   b. To each vial, add 2.9 mL Glycine Buffer Reagent, Catalog No. 332-9.
   c. Cap and invert gently several times to dissolve contents. DO NOT SHAKE.

   Using Multi-Assay Vial
   a. To NAD-ADH Multi-Assay Vial, Catalog No. 332-5, add 16.0 mL Glycine Buffer Reagent, Catalog No. 332-9.
   b. Cap and invert gently several times to dissolve contents. DO NOT SHAKE.
   c. Label test tubes for BLANK and TESTS, and add 2.9 mL NAD-ADH Solution prepared above.
2. To BLANK add 0.1 mL deionized water.
   To TEST(S) add 0.1 mL protein-free supernatant.
   Cap or cover immediately and mix by gentle inversion.
3. Allow solutions to incubate for 10 minutes at any temperature between 22°C and 37°C.
4. Transfer solutions to cuvets and measure absorbance of TESTS at 340 nm vs BLANK. Complete readings within 10 minutes.
   To determine alcohol concentration, refer to "Calculations" section.

CALIBRATION:

With spectrophotometers that demonstrate a linear response at 340 nm up to an absorbance of 1.5, alcohol concentration may be calculated directly from the absorbance at 340 nm, or alternately, may be calculated from Ethanol Standard, Catalog No. 330-20, run concurrently in the assay.

With spectrophotometers that do not yield a linear response at 340 nm, results must be derived using a standard curve as follows:

1. Assay the three standards in Ethanol Standards Set, Catalog No. 332-11, for alcohol by "Procedure 1 (Plasma, Serum and Urine)".
2. Prepare a standard curve by plotting the absorbance values vs the corresponding ethanol concentrations on graph paper.
3. The Ethanol concentration of TEST samples are then read from the standard curve.

NOTES:
1. The standard curve will not necessarily be a straight line, but should pass through the origin.
2. A new calibration curve should be prepared periodically (i.e., every 3 months), as instrument characteristics may change with time.
3. To check validity of the calibration curve, it is suggested that the 0.3% Ethanol Standard be assayed as a calibration point each day the test is performed. The absorbance value for the standard should be essentially the same as when the curve was originally established.

QUALITY CONTROL:

The reliability of test results should be monitored by routine use of control sera of known alcohol concentrations such as Ethanol Control-L, Catalog No. E 5133 and Ethanol Control-H, Catalog No. E 5258. Alcohol concentration determined by this procedure should fall within the stated ranges for the controls.

CALCULATION

The following calculations are used for spectrophotometers which demonstrate a linear response up to an absorbance of 1.5. For other instruments see "Calibration" section.

A. CALCULATIONS BASED ON ABSORBANCE AT 340 nm (A_{340})

Alcohol, mg/dL = A_{340} x 223

Where: $223 = \frac{3.01 \times 46 \times 100}{6.22 \times 0.01 \times 1 \times 1000}$

3.01	=	Total reaction volume (mL)
46	=	Molecular weight of ethanol
100	=	Conversion of mL to dL
6.22	=	Millimolar absorptivity of NADH at 340 nm
0.01	=	Volume of sample (mL)
1	=	Lightpath of cuvet (cm)
1000	=	Conversion of mL to liter

Example: A_{340} = 0.55

Alcohol, mg/dL = 0.55 x 223 = 123

B. CALCULATIONS BASED ON ETHANOL STANDARD (0.08%), CATALOG NO. 330-20

Alcohol Concentration =

$\frac{A_{340}\ \text{Sample}}{A_{340}\ \text{Standard}}$ x Concentration of Ethanol Standard

Alcohol, mg/dL = $\frac{A_{340}\ \text{Sample}}{A_{340}\ \text{Standard}}$ x 80

C. CONVERSION FACTORS:

To convert mg/dL to % (w/v), divide mg/dL results by 1000. Thus, 123 mg/dL is equivalent to 0.123% (w/v).

To convert results to SI units (mmol/L), multiply mg/dL results by 0.217. Thus, 123 mg/dL is equivalent to 26.7 mmol/L.

LIMITATIONS

If alcohol concentration in the sample exceeds 300 mg/dL, dilute 1 part sample with 1 part isotonic saline and reassay. Multiply the result by 2 to compensate for dilution.

EXPECTED VALUES

Ethanol levels in abstaining subjects are not detectable by most enzymatic or chromatographic methods.[7] From a study of over 6000 subjects, indications are that very few people are intoxicated at blood ethanol levels of 0.05%.[8] At levels of 0.15% or over, more than 50% of individuals are grossly intoxicated. Practically all persons are inebriated at ethanol concentrations above 0.35%.

Serum alcohol concentrations are usually about 16% higher than blood samples from which they are derived.[9] However, the ratio of serum to blood alcohol levels may vary due to variable water content of blood samples.

PERFORMANCE CHARACTERISTICS[5]

REPRODUCIBILITY STUDIES:

Replicate assays of two "ethanol spiked" serum pools yielded mean ethanol values of 0.071% and 0.186%. Standard deviations were found to be 0.002 and 0.0029 and the coefficients of variation were 4.1% and 1.08%, respectively.

CORRELATION STUDIES:

A series of 47 samples of serum, plasma and blood having ethanol values ranging from 0.0066 – 0.292% was assayed by the described technique and the former Sigma method (Procedure No. 331-UV). The two techniques yielded respective means of 0.1229% and 0.1239% with a correlation coefficient of 0.998.

In a correlation study by Poklis and Mackell,[10] 100 samples compared by the Sigma procedure (x), and a gas chromatography technique (y), yielded a correlation coefficient of 0.990. The regression equation was y = 1.03x – 0.0584 mg/dL.

In another study, 21 blood samples having ethanol values ranging from 0.046 – 0.263% were determined by the described procedure (y) and by gas chromatography (x). Statistical analysis of values obtained revealed a correlation coefficient of 0.988. The regression equation was y = 0.978x – 0.76 mg/dL.

RECOVERY STUDIES:

Ethanol was added to pooled sera, producing levels of 0.080, 0.105 and 0.140%. Recoveries were 97.5, 99.0 and 98.5%, respectively.

REFERENCES

1. Bucher T, Redetzki H: Eine Spezifische Photometrische Bestimmung von Athylalkohol auf Fermentativen Wege. Klin Wochenschr 29:615, 1951
2. Lundquist F: The Determination of Ethyl Alcohol in Blood and Tissues. IN Methods of Biochemical Analysis, Vol VII, D Glick, Editor, Interscience, New York, 1957, pp 217-251
3. Jones D, Gerber LP, Drell W: A rapid enzymatic method for estimating ethanol in body fluids. Clin Chem 16:402, 1970
4. Hepler OE: Manual of Clinical Laboratory Methods, 4th ed., CC Thomas, Springfield (IL), 1949, p 329
5. Data obtained by Sigma Diagnostics
6. Young DS, Pestaner LC, Gibberman V: Effects of drugs on clinical laboratory tests. Clin Chem 21:1D, 1975
7. Dubowski KM: Alcohol analysis: Clinical laboratory aspects. Part II. Laboratory Management, April, 1982, p 27
8. American Medical Association: Alcohol and the Impaired Driver, 1968
9. Shoemaker MJ: Blood alcohol determination. Pathologist, February, 1985, p 6
10. Poklis A, Mackell MA: Evaluation of a modified alcohol dehydrogenase assay for the determination of ethanol in blood. Clin Chem 28:2125, 1982

REAGENTS FOR DETERMINATION OF ALCOHOL (ETHANOL)

KITS

Catalog No.	**332-A**	**332-B**	**332-C**
Maximum Assays	25	100	100
Contents – Catalog Numbers			
NAD-ADH, 330-1	25x3 mL	100x3 mL	—
NAD-ADH, 332-5	—	—	20x16 mL
Ethanol Standard, 330-20	5 mL	2x5 mL	2x5 mL
Glycine Buffer, 332-9	110 mL	3x110 mL	3x110 mL

INDIVIDUAL REAGENTS

Catalog No.	**Item**	**Quantity**
330-1	NAD-ADH SINGLE ASSAY VIAL	10x3 mL 15x3 mL
	NAD-ADH MULTI-ASSAY VIAL	
332-5		10x16 mL
332-30		100 mL
330-20	ETHANOL STANDARD SOLUTION	5 mL
332-9	GLYCINE BUFFER REAGENT	110 mL 500 mL
331-7	TRICHLOROACETIC ACID SOLUTION	50 mL 200 mL

OPTIONAL REAGENTS

Catalog No.	**Item**	**Quantity**
E 5133	ETHANOL CONTROL-L	6x2 mL
E 5258	ETHANOL CONTROL-H	6x2 mL
332-11	ETHANOL STANDARDS SET Set contains 2x3 mL each of aqueous standards with ethanol concentrations of 0.05, 0.10 and 0.30% (w/v). Standards available in set only and cannot be ordered individually.	6x3 mL

Procedure No. 332-UV
Previous Revision: May 1995
Revised: February 1996

NOTES

SIGMA DIAGNOSTICS
P.O. BOX 14508 ST. LOUIS, MO 63178 USA
Technical Services: 1-800-325-0250 or call collect 314-771-3122
To Order: 1-800-325-3010 or call collect 314-771-5750

UNITED KINGDOM
SIGMA-ALDRICH COMPANY LTD. DIAGNOSTICS DIVISION
FANCY ROAD, POOLE, DORSET BH12 4QH
Linkline: 0800 373731