Ethanol Colorimetric Assay Kit

Item No. 702260

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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
400531	Ethanol Assay Buffer	1 vial/30 ml	4°C
400532	Ethanol Standard	1 vial/500 µl	RT
400533	Ethanol Assay Probe	1 vial/240 µl	-20°C
400534	Ethanol Assay Enzyme Mix	1 vial/150 µl	-20°C
700020	Half-Volume 96-Well Clear Plate	1 plate	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Ethanol Colorimetric Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 570 nm
2. Non-adhesive foil
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. 1 M potassium phosphate buffer, pH 7.5 (if testing vinegars)

INTRODUCTION

Background

Ethanol is a product of fermentation by yeast and certain bacteria and is important in the production of wine, alcoholic beverages, and fuel alcohol.[1,2] Ethanol is produced by the yeast *S. cerevisiae via* fermentation when glucose levels are high under aerobic conditions, an effect known as the Crabtree effect.[1] When yeast are grown on galactose instead of glucose, the Crabtree effect is circumvented and the amount of ethanol produced can be used as an indication of increased glycolysis and potential mitochondrial dysfunction.[1,3]

About This Assay

Cayman's Ethanol Colorimetric Assay Kit provides a colorimetric-based method for measuring ethanol in alcoholic beverages, vinegars, and synthetic media for yeast culture. In the assay, an alcohol oxidase (AOX) oxidizes ethanol, producing acetaldehyde and hydrogen peroxide (H_2O_2). The Ethanol Assay Probe is oxidized by H_2O_2 in the presence of horseradish peroxidase (HRP), generating a product with absorbance that can be measured at 570 nm. This assay has a range of 0-4 mM (0-0.02% v/v) and a limit of detection (LOD) of 0.01 mM.

Principle Of This Assay

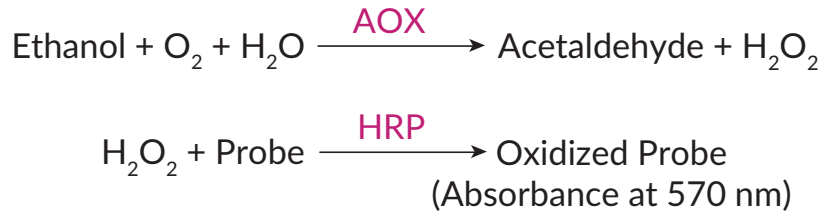


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Ethanol Assay Buffer - (Item No. 400531)**

   This vial contains 30 ml of Ethanol Assay Buffer, pH 7.5, which is ready to use as supplied. Thaw at room temperature prior to use. The Ethanol Assay Buffer will be stable for at least two months when stored at 4°C.

2. **Ethanol Standard - (Item No. 400532)**

   This vial contains 500 µl of ethanol, which is ready to use as supplied. The cap should be tightened after each use to prevent evaporation.

3. **Ethanol Assay Probe - (Item No. 400533)**

   This vial contains 240 µl of Ethanol Assay Probe in DMSO. Thaw at room temperature prior to use. The probe is sensitive to oxygen and light. Mix gently and keep the cap tightened when not in use. One vial provides a sufficient volume to assay 100 wells. If not assaying an entire 96-well plate, prepare aliquots and store at -20°C. The probe will be stable for at least two months when stored at -20°C.

4. **Ethanol Assay Enzyme Mix - (Item No. 400534)**

   This vial contains 150 µl of Ethanol Assay Enzyme Mix in a formulated storage buffer. Thaw on ice prior to use. One vial provides a sufficient volume to assay 100 wells. If not assaying an entire 96-well plate, prepare aliquots and store at -20°C. The enzyme mix will be stable for at least one month when stored at -20°C.

Sample Preparation

Liquor

Given that the density of pure ethanol is 0.785 g/cm^3 at 25°C, the concentration of pure ethanol is ~17.04 M.[5] Typically, liquors contain 5-40% ethanol. It is recommended to dilute liquor samples 250- to 2,000-fold with Ethanol Assay Buffer prior to the assay.

Vinegar

Vinegar of different types may contain disparate levels of antioxidants, which interfere with this assay. It is recommended to dilute the vinegar of interest at least 5-fold with 1 M potassium phosphate buffer, pH 7.5 (not provided), which neutralizes the sample without interfering with this assay. Prepare 2-fold serial dilutions of the neutralized vinegar with 1 M potassium phosphate buffer, pH 7.5, prior to the assay. Perform the assay and detemine the absorbance value of each dilution. If each consecutive dilution absorbance value (background subtracted) decreases by approximately 2-fold from the previous dilution's absorbance value (background subtracted), the sample is suitable for the assay.

Synthetic Yeast Culture Medium

To analyze ethanol secreted by yeast cultured in a synthetic medium, collect supernatant after centrifugation. Dilute the supernatant with Ethanol Assay Buffer to fall within the range of the standard curve.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in triplicate is shown in Figure 2, below. It is suggested that the contents of each well are recorded on the template sheet provided (see page 21).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	3	3	3	11	11	11	19	19	19
B	S2	S2	S2	4	4	4	12	12	12	20	20	20
C	S3	S3	S3	5	5	5	13	13	13	21	21	21
D	S4	S4	S4	6	6	6	14	14	14	22	22	22
E	S5	S5	S5	7	7	7	15	15	15	23	23	23
F	S6	S6	S6	8	8	8	16	16	16	24	24	24
G	1	1	1	9	9	9	17	17	17	25	25	25
H	2	2	2	10	10	10	18	18	18	26	26	26

S1-S6 = Standard Wells
1-26 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume in the assay is 120 µl in all of the wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicate is preferred), but it is at the user's discretion to do so.
- The assay is performed at room temperature except for the incubation portion, which is performed at 37°C.
- Monitor the absorbance at 570 nm.

Standard Preparation

Dilute 50 µl of the Ethanol Standard with 800 µl of Ethanol Assay Buffer to yield a 1 M bulk standard. Transfer 40 µl of the 1 M bulk standard to 960 µl of Ethanol Assay Buffer to yield a 40 mM bulk standard. Obtain six clean tubes (or one dilution plate) and label the tubes/wells S1 through S6. Pipette 180 µl of Ethanol Assay Buffer to tube/well S1. Pipette 100 µl of Ethanol Assay Buffer to tubes/wells S2-S6. Transfer 20 µl of the 40 mM bulk standard to tube/well S1. Mix well. Transfer 100 µl from tube/well S1 to S2, and mix well. Then, transfer 100 µl from tube/well S2 to S3 and mix well. Repeat this process for tubes/wells S4-S5. Do not add any ethanol to S6. Keep the tubes/plate containing standards capped/sealed until ready to use.

The bulk standards will be stable at room temperature for at least five hours if the container is kept closed. New standards should be prepared for each experiment.

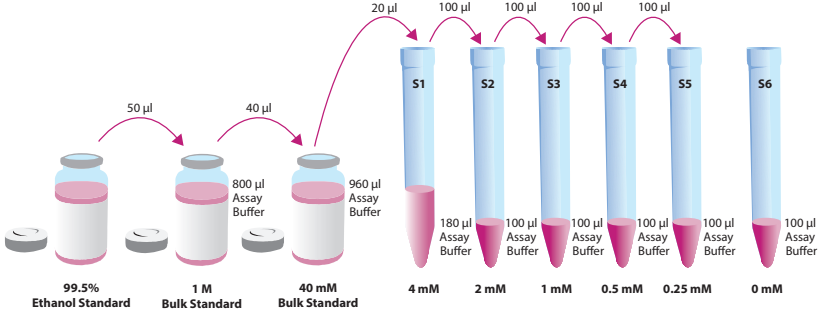


Figure 3. Preparation of the ethanol standards

Performing the Assay

1. **Reaction Mix**

   The Ethanol Assay Enzyme Mix is supplied as an 80X solution. To prepare the reaction mix, combine the Ethanol Assay Buffer and Ethanol Assay Enzyme Mix at a 79:1 ratio. Use the reaction mix within 10 minutes of preparation.

   Calculate the amount of reaction mix to be prepared using the following formula:

   (# sample wells + # standard wells) x 80 µl x 1.2 = volume (µl) of reaction mix to prepare

   e.g. Combine 9,480 µl Ethanol Assay Buffer and 120 µl Ethanol Assay Enzyme Mix for assaying 100 wells.

2. Add 80 µl of reaction mix to all wells to be used.

3. **Standard Wells**

   Add 20 µl of standard (S1-6) per well in the designated wells on the plate (see **Sample plate format**, Figure 2, page 10). Mix by pipetting up and down.

4. **Sample Wells**

   Add 20 µl of sample to at least two wells. Mix by pipetting up and down. When necessary, samples should be neutralized to pH 7.5 prior to dilution (see **Sample Preparation** on page 9).

5. Gently wrap the plate with non-adhesive foil to protect from light and incubate for 20 minutes at 37°C.

6. **Diluted Ethanol Assay Probe**

   The Ethanol Assay Probe is supplied as a 16X solution. To prepare the probe at its working concentration, combine the Ethanol Assay Buffer and Ethanol Assay Probe at a 15:1 ratio. Use the diluted probe within 15 minutes of preparation. Protect from air and light until ready to use.

   Calculate the amount of diluted Ethanol Assay Probe to be prepared using the following formula:

   (# sample wells + # standard wells) x 20 µl x 1.2 = volume (µl) of diluted Ethanol Assay Probe to prepare

   e.g. Combine 2,250 µl Ethanol Assay Buffer and 150 µl Ethanol Assay Probe for assaying 100 wells.

7. Quickly add 20 µl of the diluted Ethanol Assay Probe to all wells being used, including the sample and standard wells. Mix by pipetting up and down without generating bubbles.
8. Shake for 15 seconds and read absorbance at 570 nm (A_{570}).

Important Notes:

If assaying more than one plate, plates should not be stacked during incubation.

The reaction does not stop after the addition of the diluted Ethanol Assay Probe. Extending incubation times at any step of this assay is not recommended.

This kit is highly sensitive to short-chain alcohols. The absorption of alcohol vapors by the kit components may cause a very high background (A_{570} of S6 > 0.4). It is critical to perform the assay in a clean environment.

ANALYSIS

Calculations

1. Determine the average absorbance of each standard and sample.
2. Subtract the average absorbance value of the zero-point standard (S6) from itself and all other standards and samples to obtain corrected signal (CS) values.
3. Plot the CS values of each standard as a function of the ethanol concentration of the standard. See Figure 4, on page 16, for a typical standard curve.
4. Calculate the ethanol concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the CS for each sample.

$$\text{ethanol (mM)} = \left[\frac{\text{CS - (y-intercept)}}{\text{slope}} \right] \text{x dilution factor}$$

Starting Unit	Factor	Final Unit
mM	0.005869	% v/v
mM	4.607	mg/dl
% v/v	170.4	mM

Table 1. Unit conversion. To determine the final unit, multiply the starting unit by the factor in column 2.

Performance Characteristics

Sensitivity

The lower limit of quantification (LLOQ) of the assay is 0.06 mM.

The lower limit of detection (LOD) of the assay is 0.01 mM.

Precision

When a series of 24 neutralized white vinegar measurements were performed on the same day, the intra-assay coefficient of variation was 14%. When a series of six neutralized white vinegar measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 11%.

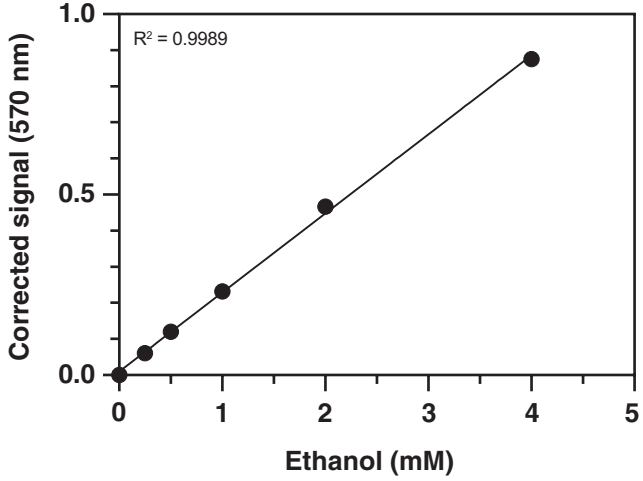


Figure 4. Ethanol standard curve

Interferences

The interferences of this assay include short-chain alcohols such as methanol, propanol, butanol, and allyl alcohol.[5] Other interferences include antioxidants, reducing reagents, and oxidizing reagents. Reagents that affect the pH of the reaction also interfere with this assay.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/ technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Ethanol concentration above the highest point in the standard curve	The ethanol concentration is too high in the sample	Dilute samples with Ethanol Assay Buffer and re-assay *NOTE: Remember to account for the dilution factor when calculating the ethanol concentration*
Exceptionally high absorbance of the zero-point standard (S6)	A. Short-chain alcohols present in the working environment or incubator B. Ethanol Assay Probe exposed to heat, air, or light during storage	A. Re-assay after examining and eliminating any sources of short-chain alcohols in the working environment B. Repeat the assay with a new Ethanol Assay Probe

References

1. De Deken, R.H. The Crabtree effect: A regulatory system in yeast. *J. Gen. Microbiol.* **44(2)**, 149-159 (1966).
2. Henderson, C.M., Lozada-Contreras, L., Jiranek, V. *et al.* Ethanol production and maximum cell growth are highly correlated with membrane lipid composition during fermentation as determined by lipidomic analysis of 22 *Saccharomyces cerevisiae* strains. *Appl. Environ. Microb.* **79(1)**, 91-104 (2013).
3. Ji, J., Damschroder, D., Bessert, D. *et al.* NAD supplementation improves mitochondrial performance of cardiolipin mutants. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1867(4)**, 159094 (2022).
4. Dienys, G., Jarmalavičisu, S., Budrienė, S. *et al.* Alcohol oxidase from the yeast *Pichia pastoris* - a potential catalyst for organic synthesis. *J. Mol. Catal. B: Enzymatic* **21(1-2)**, 47-49 (2003).
5. Williams, M.L. CRC Handbook of Chemistry and Physics. (2004).

Prepare Reaction Mix:
Buffer:Enzyme Mix = 79:1

Add 80 µl Reaction Mix
*to **Standard Wells** and **Sample Wells***

↓

Add 20 µl Standard or Sample
*to **Standard Wells** and **Sample Wells***

↓ 37°C, 20 minutes, in the dark
While waiting, **prepare Diluted Probe:**
Buffer:Probe = 15:1

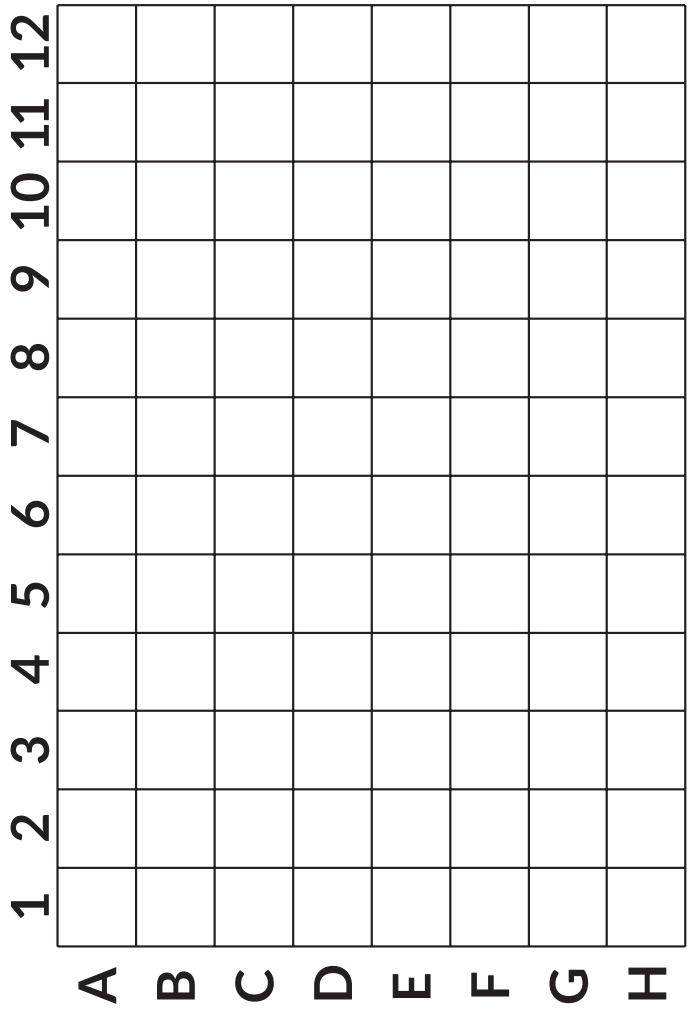
Add 20 µl Diluted Probe
*to all **Wells***

↓ Shake 15 seconds

↓

A_{570}

Figure 5. Assay summary



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.