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Enzymatic Ethanol Assay

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ABSTRACT

This protocol describes a 96-well-plate-based, enzymatic assay for reliably estimating ethanol concentrations in experimental samples in one hour. In the presence of excess NAD^+, alcohol dehydrogenase (ADH) is employed to convert ethanol to acetaldehyde. The concomitant conversion of NAD^+ to NADH is monitored via increased absorbance at 340 nm. When highly accurate analytical techniques (such as high performance liquid chromatography) are not necessary, or are too costly or low-throughput, this assay offers reliable, inexpensive, and rapid detection of ethanol concentrations. This assay is useful for applications such as determining relative ethanol production from microbial fermentations, and detecting ethanol evaporation from media.

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KEYWORDS

alcohol dehydrogenase, assay, ethanol, 96-well plate

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MATERIALS TEXT

Reagents

Sodium pyrophosphate decahydrate **Sigma Aldrich Catalog #221368**

Glycine **Sigma Aldrich Catalog #G7126**

Semicarbazide hydrochloride **Sigma Aldrich Catalog #S2201**

Hydrochloric Acid Solution, 1N **Fisher Scientific Catalog #SA48-1**

β-Nicotinamide adenine dinucleotide hydrate **Sigma Aldrich Catalog #N6522**

This specific vendor and catalog number are recommended for β-Nicotinamide adenine dinucleotide hydrate (NAD^+) to avoid solubility issues.

Potassium phosphate dibasic **Fisher Scientific Catalog #P288**

Potassium phosphate monobasic **Fisher Scientific Catalog #P380**

Bovine serum albumin **Sigma Aldrich Catalog #A3059**

Alcohol dehydrogenase enzyme **Sigma Aldrich Catalog #A3263**

Ethyl alcohol, 200 proof, anhydrous, ≥99.5% **Sigma Aldrich Catalog #459836**

Clear 96-well flat-bottom microplate **Corning Catalog #353072**

ThermalSeal RTS sealing film **Sigma Aldrich Catalog #Z742256**

Several sealing films were tested during protocol optimization. The ThermalSeal RTS sealing film was the highest performing seal for this application, consistently preventing ethanol evaporation.

General Supplies and Equipment

- Laboratory balance
- P1000, P200, P20, and P10 pipettes and corresponding pipette tips
- pH meter
- 1.5-mL microcentrifuge tubes
- -80°C and -20°C Freezers
- Repeater pipette
- 5 mL repeater pipette tip
- Multi-channel pipette (8-channel) capable of transferring 10 µL
- Microplate spectrophotometer

Protocol was developed with BioTek PowerWave XS microplate spectrophotometer.

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Preparation

1 Create a microplate spectrophotometer program to read absorbance at 340 nm of each well in a 96-well plate.

- The program should take absorbance readings at 340 nm every 20 - 30 seconds (or at minimum interval), shaking for 10 seconds immediately before each reading.
- The program should be set to run for 1 hour at **30 °C** .

2 Make nicotinamide adenine dinucleotide (NAD^+) stock solution

Component	Concentration	Amount
NAD	50 mM	0.4976 g
Water	---	Up to 15 mL

Vortex to ensure that NAD^+ is completely dissolved.

Store aliquots in 1.5-mL microcentrifuge tubes at **-80 °C** .

3 Make pH**9.0** glycine buffer

Component	Concentration	Amount
Sodium pyrophosphate	33.3 g/L	3.333 g
Glycine	1.67 g/L	0.167 g
Semicarbazide hydrochloride	0.125 g/L	12.5 mg
Hydrochloric acid (1N)	---	To pH 9.0
Water	---	Up to 100 mL

Store at **25 °C** for up to 1 month.

4 Make alcohol dehydrogenase (ADH) enzyme stock solution

Component	Concentration	Amount
Potassium phosphate dibasic	83 g/L	1.66 g
Potassium phosphate monobasic	17 g/L	0.34 g
Bovine serum albumin	1 g/L	0.02 g
Alcohol dehydrogenase enzyme (~300 U/mg)	20 U/mL	1.36 mg
Water	---	Up to 20 mL

Store aliquots in 1.5-mL microcentrifuge tubes at **-20 °C** for up to 1 month.

Experimental Steps

5 Make ethanol standards at eight concentrations encompassing the range of concentrations expected from the experimental samples, including a 0 g/L ethanol standard. Dilute ethanol in the same media present in the experimental samples to make the standards.

> This protocol was optimized for detecting ethanol concentrations between 0.05 and 1.5 g/L in the assay solution, which corresponds concentrations between 1 and 30 g/L in the experimental samples. Measuring ethanol concentrations outside of this range will require preparing a different standard curve, and making different dilutions of samples (while maintaining 200 µL total volume in each well) to achieve final ethanol concentrations

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between 0.05 and 1.5 g/L in the assay solution.

6 Make master mix for enzyme assay on ice, adding components in the order listed in the table below. If NAD^+ and ADH stock solution aliquots are frozen, defrost the necessary volume on ice before proceeding.

The master mix in the table below is sufficient for one full 96-well plate (with each well containing 190 µL of assay master mix). If running more or less than one plate, scale the recipe accordingly.

Component	Final Concentration	Volume
NAD stock solution	8 mM	3.2 mL
ADH stock solution	0.1 U/mL	111 uL
Glycine buffer	---	Up to 20 mL

Keep the master mix on ice during and after preparation.

7 Turn on the microplate spectrophotometer and open the program (defined in Step 1) to begin heating to **30 °C** , the temperature at which the assay will be run.

8 Fill all wells of a 96-well plate with 190 µL of the assay master mix using a repeater pipette and a 5 mL repeater pipette tip.

9

Work as quickly as possible through the next three steps (Steps 9 -11) in order to minimize ethanol evaporation and substantial progression of the enzymatic reaction before the microplate program has started.

Designate two of the 12 columns in the 96-well plate for standards, and add 10 µL of each standard with a P10 pipette to the 190 µL of assay master mix. Add the standards in order of either increasing or decreasing concentration down the columns.

10 To each well in the remaining 10 columns of the 96-well plate, add 10 µL of experimental sample to the 190 µL of assay master mix. It is recommended to use a multi-channel (8-channel) pipette for this step to fill the plate as rapidly as possible.

At this point, the assay has started and the plate should be read as quickly as possible.

11 Seal the plate with a ThermalSeal RTS sealing film. Use a sealing film roller or a roll of tape to ensure that the film is adhered closely to the rim of each well, taking care to avoid any large wrinkles or gaps.

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12 Place the sealed 96-well plate (without a lid) in the microplate spectrophotometer (preheated to 🌡 **30 °C**) and start the program to read the absorbance at 340 nm. 1h

As alcohol dehydrogenase converts NAD^+ to NADH, absorbance at 340 nm will increase with time. If after 30 minutes, the absorbance at 340 nm plateaus (is no longer increasing) for all of the samples and standards, the program can be terminated. If not, let the program run for the full hour.

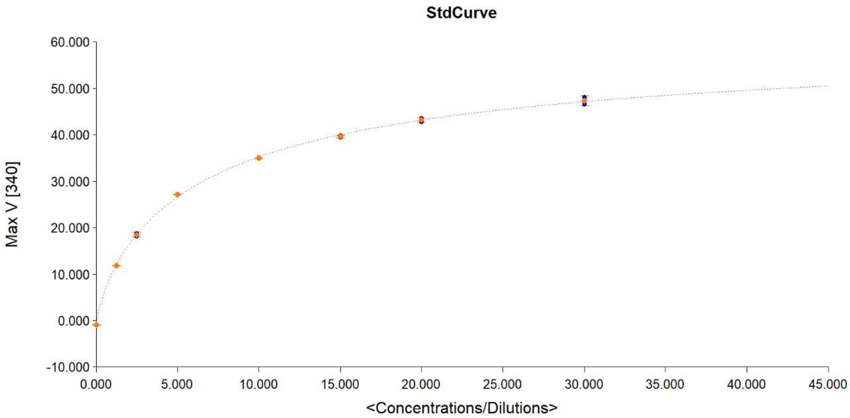
Data Analysis

13 Use the 340 nm absorbance data to calculate Vmax (change in absorbance per unit time) for each well in the plate, including wells containing experimental samples and standards. Use at least 30 data points in a range where absorbance is linearly increasing with time to calculate Vmax.

14 Use the standard wells with known ethanol concentrations to generate a standard curve, as specified in the sub-steps below. Sample data is attached. **Sample Data_2021_02_09.xlsx**

14.1 User-defined standard concentrations will serve as x-axis data inputs. Vmax data at 340 nm will serve as y-axis data inputs.

14.2 Use a four-parameter nonlinear regression curve fit to generate a standard curve with the formula Y = (A-D)/(1+(X/C)^B)+D.



A representative standard curve, relating Vmax (mOD/min) at 340 nm, denoted here as Max V [340], and ethanol concentrations (g/L).

15 Use the standard curve to calculate ethanol concentrations in the experimental samples.

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