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Enzymatic Ethanol Assay V.2

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We use this protocol and it's working

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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.

Version notes:
10-27-2021. Add 50 ul water to the assay plate first, then add sample. This prevents ethanol evaporation and allows the assay to be started (by adding assay master mix) immediately before reading.

Materials

Reagents

- Sodium pyrophosphate decahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**221368
- Glycine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G7126
- Semicarbazide hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2201
- Hydrochloric Acid Solution, 1N **Fisher Scientific Catalog #**SA48-1
- β-Nicotinamide adenine dinucleotide hydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**N6522

Note

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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3059
- Alcohol dehydrogenase enzyme **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3263
- Ethyl alcohol, 200 proof, anhydrous, ≥99.5% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**459836
- Clear 96-well flat-bottom microplate **Corning Catalog #**353072
- ThermalSeal RTS sealing film **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**Z742256

Note

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

Note

Protocol was developed with BioTek PowerWave XS microplate spectrophotometer.

Troubleshooting

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

	Component	Concentration	Amount
	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.

Note

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 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.

Note

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

	Component	Final Concentration	Volume
	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 50 µL of water. The purpose of the water is to minimize ethanol evaporation during subsequent sample pipetting steps.

9

Note

Work as quickly as possible through the next three steps (Steps 9 -12) 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.

In the remaining wells, add 10 µL of each sample. It is recommended to use a multi-channel (8-channel) pipette for this step to fill the plate as rapidly as possible.

10 Start the assay by adding 190 µL of the assay master mix using a repeater pipette and a 5 mL repeater pipette tip.

Note

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.

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

Note

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

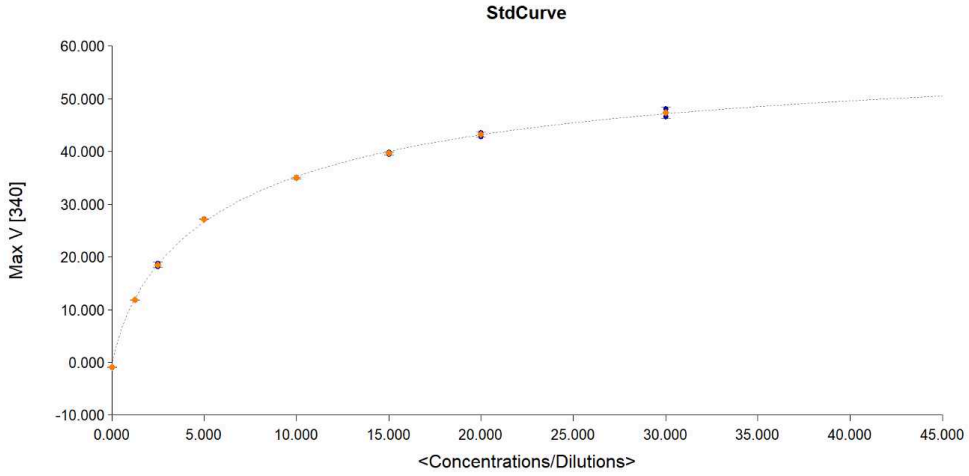
13 Use the 340 nm absorbance data to calculate Vmax (change in absorbance per unit time) for each well in the plate, including wells containing experimental samples and standards. Use at least 30 data points in a range where absorbance is linearly increasing with time to calculate Vmax.

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.