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# Acetaldehyde Quantification in Microbial Fermentation Samples

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**Protocol status:** Working

**We use this protocol and it's working**

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**Keywords:** Acetaldehyde quantification, Hydrazine derivatization, Biological fermentation, HPLC, acetaldehyde quantification in microbial fermentation sample, acetaldehyde quantification, concentration of acetaldehyde, microbial fermentation sample, acetaldehyde, hydrazine derivatization reaction, microbial, dinitrophenylhydrazine, using high performance liquid chromatography, high performance liquid chromatography

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## Abstract

This protocol details a broadly applicable approach for quantifying the concentration of acetaldehyde present in a microbial fermentation sample. To quantify acetaldehyde, a hydrazine derivatization reaction is performed following sampling using 2,4-dinitrophenylhydrazine (2,4-DNPH), which reacts with acetaldehyde in a 1:1 stoichiometric ratio to produce acetaldehyde-2,4-DNPH, a stable product at room temperature that can be quantified using high performance liquid chromatography (HPLC).

## Materials

### Key Reagents:

#### **2,4-dinitrophenylhydrazine (2,4-DNPH)**

Product Number: D199303; CAS: 119-26-6 (Sigma Aldrich)

[https://www.sigmaaldrich.com/US/en/product/aldrich/d199303?utm\\_source=google&utm\\_medium=cpc&utm\\_campaign=10640610201&utm\\_content=101904429261&gclid=Cj0KCQjw-r-vBhC-ARIsAGgUO2Aq0fiQYy-qIDNfqE8GBH2jazuHAm3kVKwstLlwGjex1NpBQRiXdy0aAqDbEALw\\_wcB](https://www.sigmaaldrich.com/US/en/product/aldrich/d199303?utm_source=google&utm_medium=cpc&utm_campaign=10640610201&utm_content=101904429261&gclid=Cj0KCQjw-r-vBhC-ARIsAGgUO2Aq0fiQYy-qIDNfqE8GBH2jazuHAm3kVKwstLlwGjex1NpBQRiXdy0aAqDbEALw_wcB)

#### **Acetaldehyde**

Product Number: 402788; CAS: 75-07-0 (Sigma Aldrich)

<https://www.sigmaaldrich.com/US/en/product/sial/402788>

#### **Acetonitrile**

Product Number: 34851; CAS: 75-05-8 (Sigma Aldrich)

[https://www.sigmaaldrich.com/US/en/product/sigald/34851?utm\\_source=google&utm\\_medium=cpc&utm\\_campaign=10193651927&utm\\_content=101663337533&gclid=Cj0KCQjw-r-vBhC-ARIsAGgUO2BDL9D\\_ybz\\_S42Z3yuHC5fz6z7qjNeEuAAR5Nds7CtKWSYIP6SpQlaAhKVEALw\\_wcB](https://www.sigmaaldrich.com/US/en/product/sigald/34851?utm_source=google&utm_medium=cpc&utm_campaign=10193651927&utm_content=101663337533&gclid=Cj0KCQjw-r-vBhC-ARIsAGgUO2BDL9D_ybz_S42Z3yuHC5fz6z7qjNeEuAAR5Nds7CtKWSYIP6SpQlaAhKVEALw_wcB)

#### **Pre-Derivatized Acetaldehyde-2,4-DNPH Solution**

Product Number: CRM47340; CAS: 1019-57-4 (Sigma Aldrich)

<https://www.sigmaaldrich.com/US/en/product/supelco/crm47340>

## Safety warnings

- ❗ 2,4-dinitrophenylhydrazine (2,4-DNPH) is a reactive, flammable and toxic compound. Use extreme caution and wear proper laboratory personal protective equipment (PPE) at all times when handling 2,4-DNPH.

## Introduction and Protocol Structure

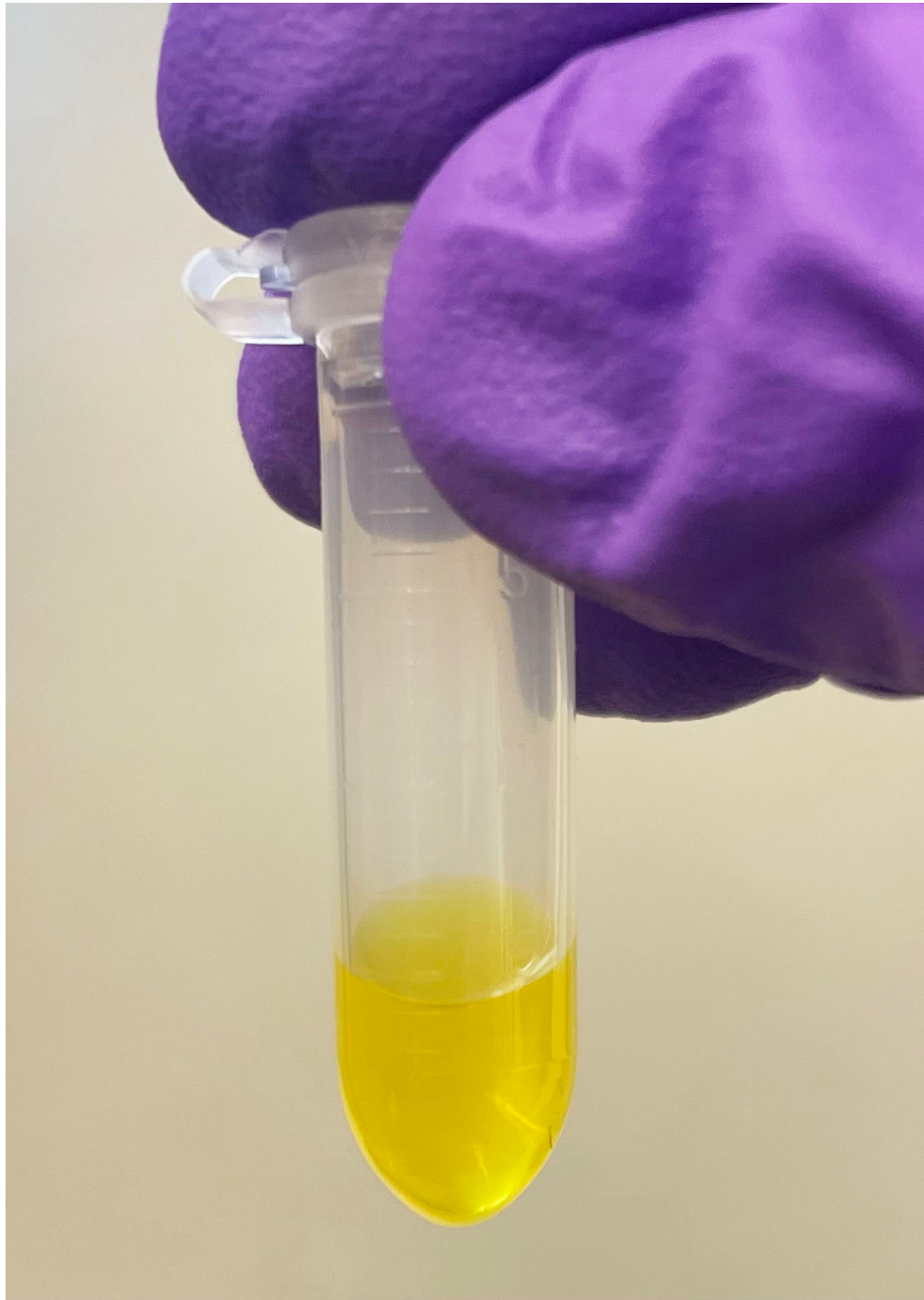
- 1 This protocol details a standardized approach for quantifying acetaldehyde in microbial fermentation samples. Accurately quantifying the concentration of acetaldehyde in biological samples poses challenges, as acetaldehyde is a volatile chemical with a boiling point of 20.2°C (68.4°F), and thus readily evaporates at room temperature (20.0–22.0°C or 68.0–72.0°F). To quantify acetaldehyde, a hydrazine derivatization reaction is performed immediately upon sampling using 2,4-dinitrophenylhydrazine (2,4-DNPH), which reacts with acetaldehyde in a 1:1 stoichiometric ratio to produce acetaldehyde-2,4-DNPH, a stable product at room temperature that can be quantified using high performance liquid chromatography (HPLC) at an absorbance of 254 nm. 
- 2 **This protocol is structured into the following sections:**
  1. Preparation of the Derivatization Reagent (Step 3 [3.1–3.11])
  2. Preparation of Acetaldehyde Standards (Steps 4–12)
  3. Derivatization of Fermentation Samples (Steps 13–24)
  4. HPLC System Setup and Method Parameters (Step 25 [25.1 – 25.10])

## Preparation of the Derivatization Reagent

- 3 The primary objective of this section of the protocol is to prepare a 100 mL stock of derivatization reagent, which consists of 0.9 g/L 2,4-DNPH and 1.0% phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) in acetonitrile.
- 3.1 Weigh out 0.09 g of 2,4-dinitrophenylhydrazine (2,4-DNPH) (red, crystalline powder; Sigma Aldrich CAS#: 119-26-6) on a scale using a small weigh boat. Note that 2,4-DNPH is highly reactive and volatile, use extreme caution and wear proper PPE at all times when handling this chemical. Record the exact mass of 2,4-DNPH weighed out in a laboratory notebook.
- 3.2 Carefully add the 0.09 g of 2,4-DNPH powder to a clean and dry 100 mL volumetric flask. The volumetric flask must be at room temperature for this step as 2,4-DNPH has explosive potential when exposed to high temperature environments. 
- 3.3 Carefully transfer the 100 mL volumetric flask containing the 2,4-DNPH powder to a fume hood.
- 3.4 In the fume hood, use a repeat pipette to add 40.0 mL of acetonitrile (Sigma Aldrich CAS#: 75-05-8) to the 100 mL volumetric flask.
- 3.5 Carefully swirl the flask to dissolve the 2,4-DNPH powder in the acetonitrile. The solution will appear as a vibrant, dark orange color following this step.



- 3.6 Add 1.1765 mL of 85.0% phosphoric acid ( $\text{H}_3\text{PO}_4$ ) to the 100 mL volumetric flask.
- 3.7 Fill the volumetric flask to the 100 mL line with acetonitrile.
- 3.8 Transfer the 100 mL of prepared derivatization reagent to a clean, dry, 200 mL glass bottle at room temperature in the fume hood. 
- 3.9 Wrap the bottle with aluminum foil to reduce exposure of the derivatization reagent to light.
- 3.10 Use tape to label the bottle as "Derivatization Reagent". Include your initials and the date prepared.
- 3.11 Store the derivatization reagent at  $-20.0^\circ\text{C}$  until use. 



**Figure 1:** This is a 500 uL aliquot of the derivatization reagent in a 2.0 mL Eppendorf tube. The derivatization reagent has golden yellow color.

## Preparation of Acetaldehyde Standards

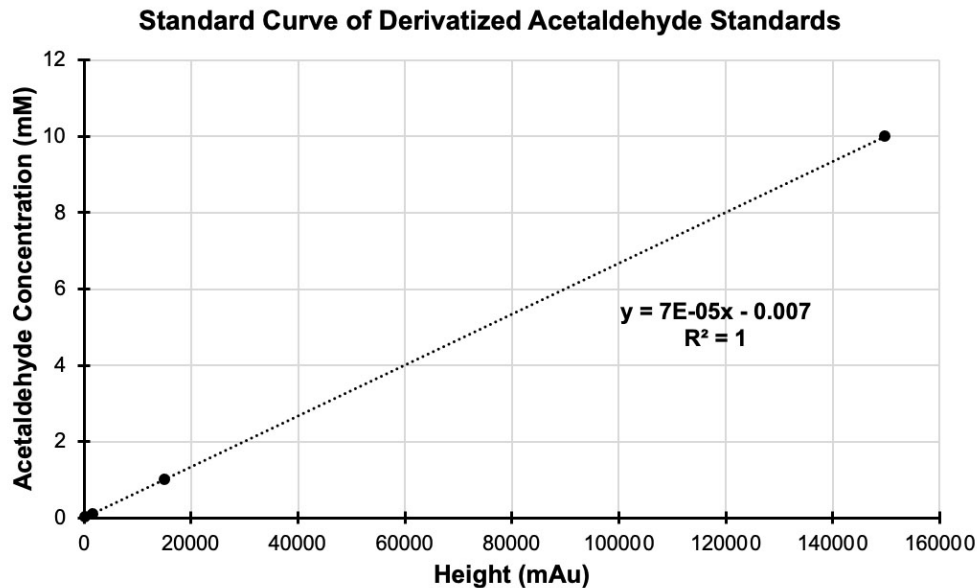
- 4 In a 4.0°C cold room, carefully break the seal of the glass vial containing pure acetaldehyde ordered from Sigma Aldrich (Sigma Aldrich CAS#: 75-07-0). Carefully pour



the 5.0 mL of pure acetaldehyde into a labeled 15 mL Falcon tube. **Note:** store acetaldehyde on ice at all times (whether working in a 4.0°C cold room or not) and store the pure acetaldehyde stock at 4.0°C when not in use to reduce evaporation.

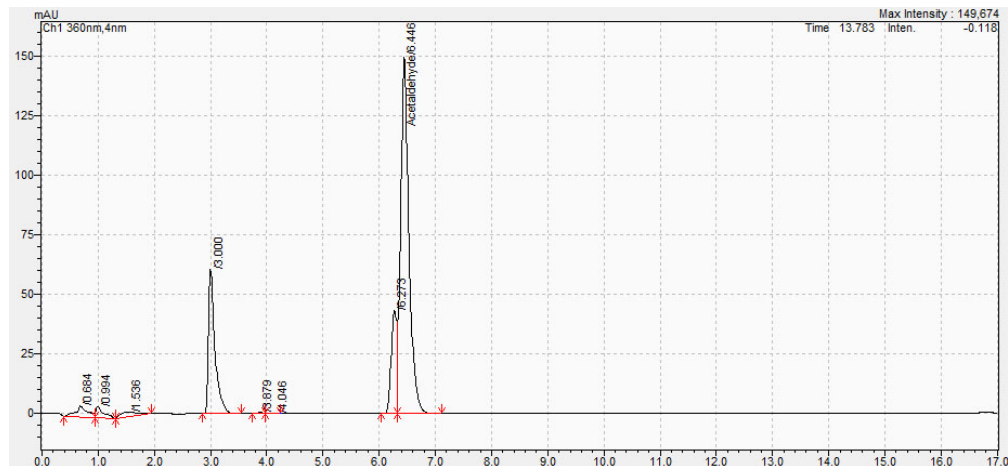
- 5 In a 4.0°C cold room, prepare 5.0 mL of a 1.0 M acetaldehyde stock solution following sub-steps 5.1-5.3:
  - 5.1 Use a repeat pipette to add 4.72 mL of MilliQ water to a 15 mL Falcon tube.
  - 5.2 Add 279.5 µL of pure acetaldehyde solution to the 4.72 mL of MilliQ water using a repeat pipette equipped with a 0.5 mL disposable plastic tip chilled to -20.0°C for 20 minutes prior to use.
  - 5.3 Following preparation, vortex the 1.0 M acetaldehyde solution for approximately 5-10 seconds to ensure complete mixing of the acetaldehyde with the MilliQ water.
- 6 Prepare 100.0 mM, 10.0 mM, 1.0 mM, 0.1 mM, and 0.01 mM acetaldehyde standards in the 4.0°C cold room following sub-steps 6.1-6.4:
  - 6.1 Using a repeat pipette equipped with a 2.5 mL disposable plastic tip, add 900 µL of MilliQ water to 5 separate 2.0 mL Eppendorf tubes labeled "100 mM", "10 mM", "1.0 mM", "0.1 mM", and "0.01 mM".
  - 6.2 Transfer 100 µL of the 1.0 M acetaldehyde stock solution to the 2.0 mL Eppendorf tube labeled "100 mM".
  - 6.3 Vortex the solution for 10 seconds to ensure thorough mixing.
  - 6.4 Perform serial dilutions to prepare the remaining stock solutions by transferring 100 µL from the "100 mM" to the 10 mM tube and so on. Vortex each tube for 10 seconds prior to performing the next dilution to ensure thorough mixing of each solution.
- 7 Following preparation of the acetaldehyde stock solutions (100 mM, 10 mM, 1.0 mM, 0.1 mM, and 0.01 mM), use a pipette to transfer 100 µL from each acetaldehyde stock solution into 500 µL of derivatization reagent in separate, labeled 2.0 mL Eppendorf tubes (final volume of each derivatized sample = 600 µL).
- 8 Vortex each derivatized acetaldehyde sample for 10 seconds.
- 9 Place and secure each of the derivatized samples on a nutating mixer in the 4.0°C cold room.

- 10 Allow the derivatized acetaldehyde samples to mix overnight on the nutating mixer for approximately 12–16 hours to ensure complete derivatization of the acetaldehyde.
- 11 Centrifuge the derivatized samples for 5.0 minutes at 2,000 rcf (x g) to pellet any residual debris.
- 12 Following mixing, transfer 200 uL of supernatant from each derivatized sample to a labeled HPLC vial and run the samples on a Shimadzu Shim-Pack XR ODS column using the methods described below under the section titled: "HPLC System Setup and Method Parameters".
- 12.1 **Note:** Although it can be run on the Shimadzu HPLC system, the 100 mM acetaldehyde standard should be omitted in the creation of a standard curve for the quantification of acetaldehyde. The reason for this is that there is not a sufficient concentration of 2,4-DNPH present in the derivatization reagent to derivatize all of the acetaldehyde present in the 100 mM standard regardless of how long the sample is mixed on the nutating mixer.
- 13 Use the height values acquired on the HPLC system (in units of mAu) for each derivatized acetaldehyde to create a standard curve of height (mAu) (x-axis) vs. concentration (mM) (y-axis). Add a trendline and the trendline equation on Excel. Use the trendline equation (in the linear form of  $y = mx + b$ ) to convert height values (in units of mAu) to concentrations (in mM) by plugging the height value in for "x" to solve for "y" (concentration of acetaldehyde in mM).

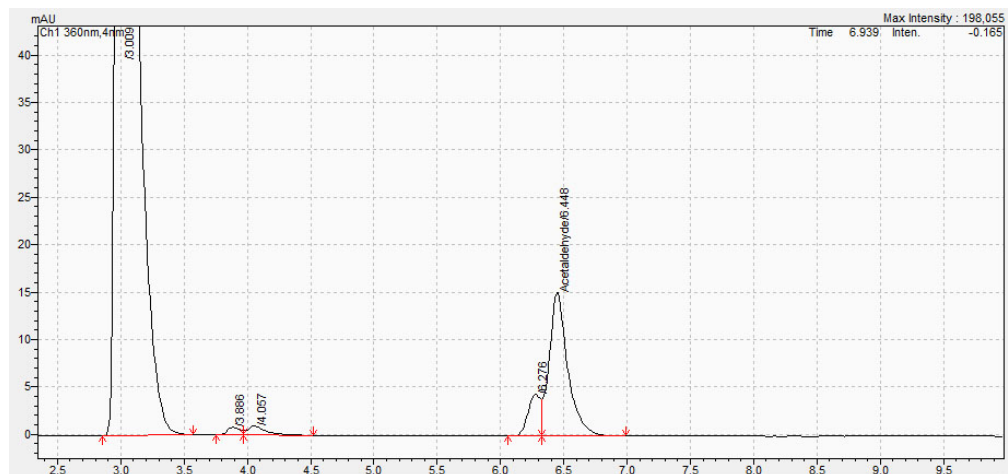


**Figure 2:** A standard curve of derivatized acetaldehyde standards (10 mM, 1.0 mM, 0.1 mM, and 0.01 mM) used to quantify the concentration of acetaldehyde (in mM) in microbial fermentation samples.

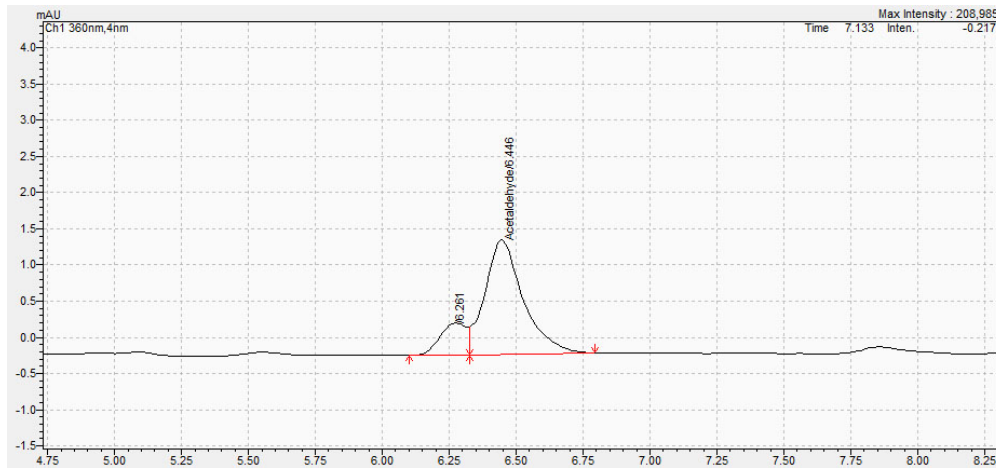
- 14 Provided below are images of the HPLC spectra from running 10 mM, 1.0 mM, 0.1 mM, and 0.01 mM derivatized acetaldehyde standards on a Shimadzu HPLC system using the setup and method parameters detailed later in this protocol under the section titled, "HPLC System Setup and Method Parameters". These derivatized acetaldehyde standards were used to produce the standard curve provided above (step 13) to quantify the concentration of acetaldehyde in microbial fermentation samples. Acetaldehyde elutes off the Shim-Pack XR ODS HPLC column at approximately 6.45 minutes as a double peak comprised of a leading short peak and a subsequent tall peak. As shown below, the short and tall acetaldehyde peaks should be split and only the tall peak should be used for the quantification of acetaldehyde. It is also important to note that 0.01 mM acetaldehyde is the limit of detection for the Shimadzu HPLC system.



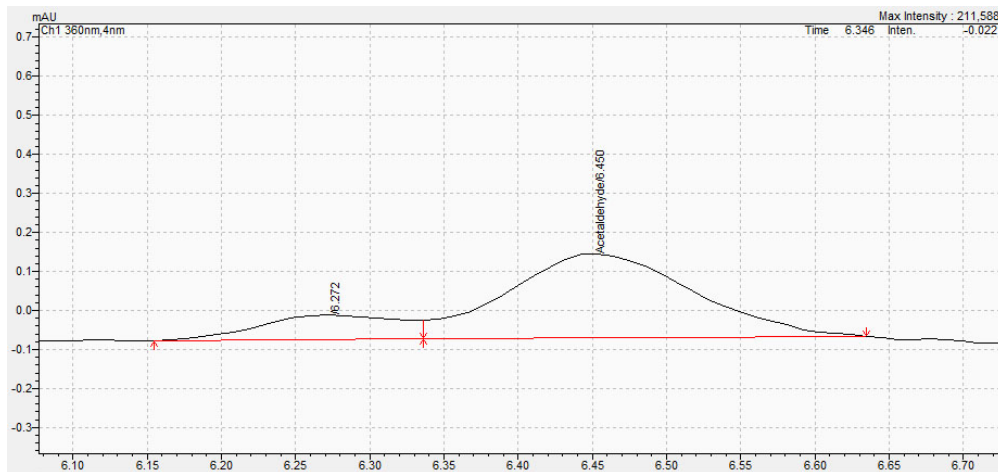
**Figure 3:** HPLC spectra for a 10 mM acetaldehyde standard.



**Figure 4:** HPLC spectra for a 1.0 mM acetaldehyde standard.



**Figure 5:** HPLC spectra for a 0.1 mM acetaldehyde standard.



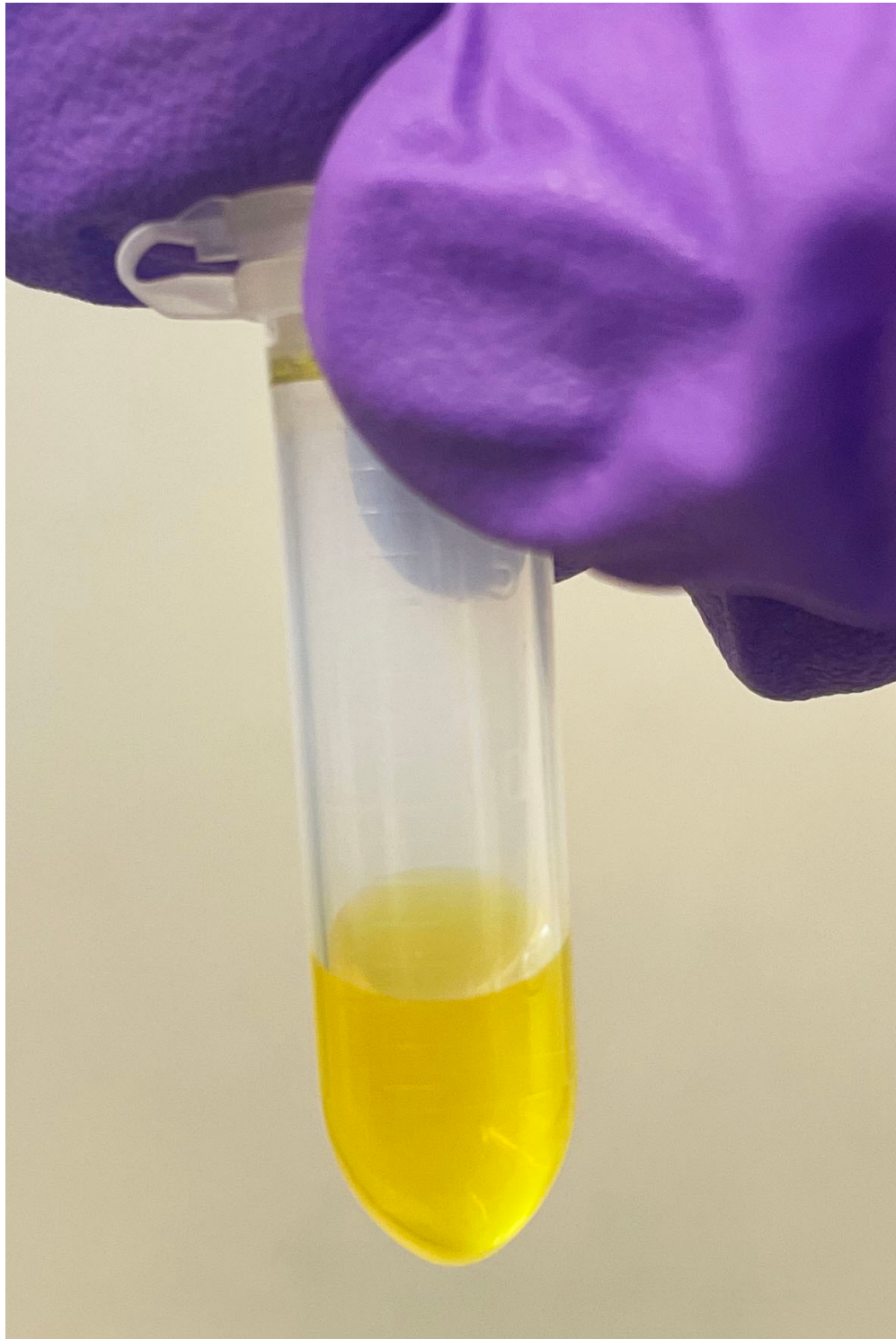
**Figure 6:** HPLC spectra for a 0.01 mM acetaldehyde standard. The Shimadzu HPLC system cannot reliably quantify acetaldehyde concentrations below 0.01 mM.

## Derivatization of Fermentation Samples

- 15 Acquire 1.0 mL of biological fermentation sample either via pipette (best for performing fermentations in Falcon tubes) or needle and syringe (best for performing fermentations in serum bottles) and transfer the 1.0 mL of sample to a labeled 2.0 mL Eppendorf tube stored on ice.
- 16 Immediately centrifuge the fermentation samples for 5.0 minutes at 21,300 rcf (x g).  
**Note:** It is best to use a centrifuge that is chilled to 4°C to reduce the potential for evaporation of acetaldehyde from the sample, however using a centrifuge that is not chilled to 4.0°C also works for this protocol.

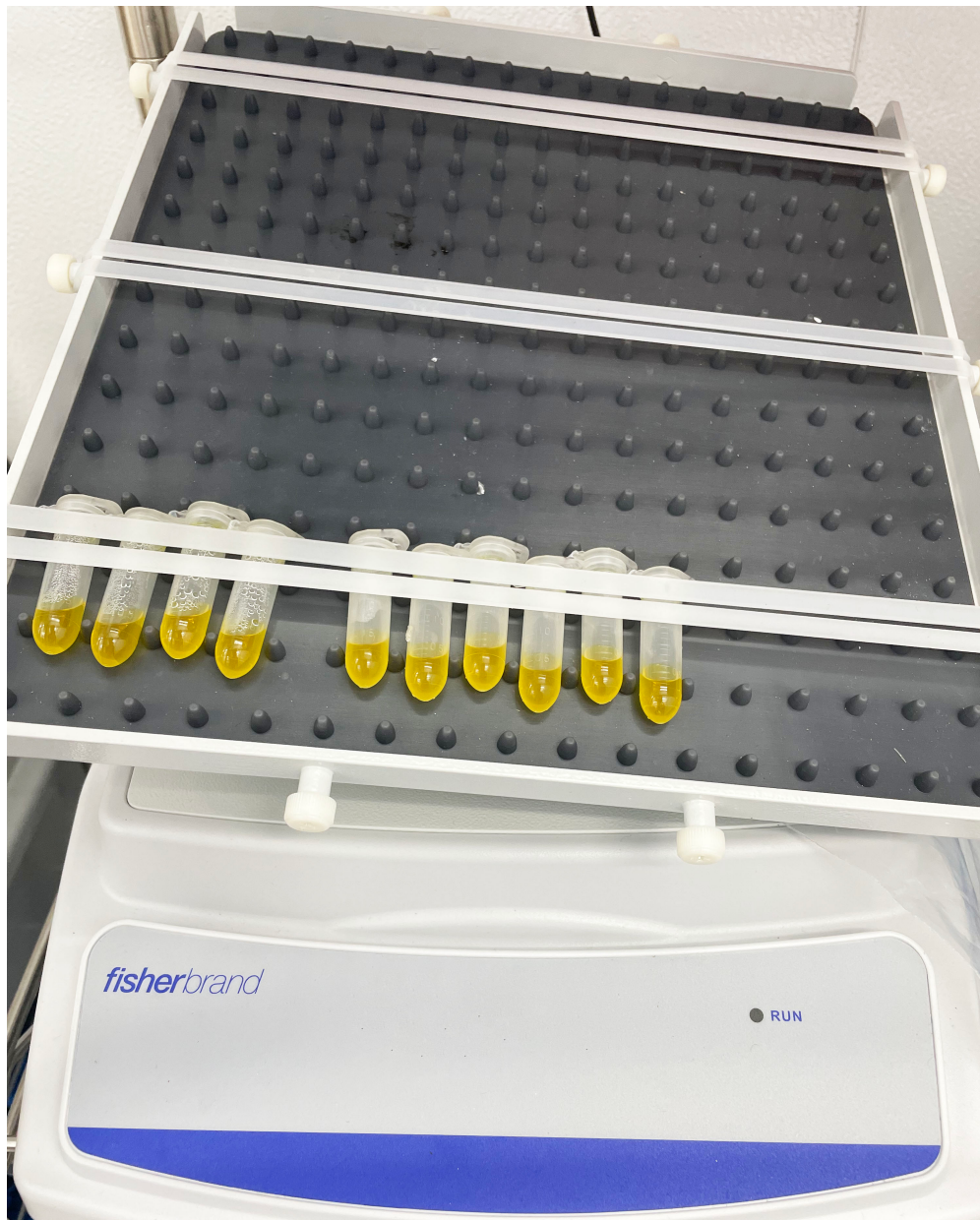


- 17 Depending on the size of the cell pellet Carefully extract 700-900  $\mu$ L of supernatant and transfer the supernatant to a labeled 2.0 mL Eppendorf tube. Store the sample on ice when not handling.
- 18 Transfer 100  $\mu$ L of fermentation sample supernatant into 900  $\mu$ L of MilliQ water (10x dilution). The purpose of this step is to ensure that there is sufficient 2,4-DNPH in the derivatization reagent to derivatize all of the acetaldehyde present in the fermentation sample. Store the sample on ice when not handling.
- 19 Immediately vortex the 10x diluted sample for 8-10 seconds to ensure thorough mixing.
- 20 Transfer 100  $\mu$ L of the 10x diluted fermentation sample into 500  $\mu$ L of derivatization reagent in a clean, dry, labeled 2.0 mL Eppendorf tube.
- 21 Immediately vortex the derivatized sample for 10 seconds to ensure thorough mixing of the acetaldehyde with the 2,4-DNPH present in the derivatization reagent.



**Figure 7:** Derivatized sample (total volume = 600  $\mu$ L) after being vortexed for 10 seconds. The sample has a slightly darker golden yellow color than the derivatization reagent.

- 22 Transfer the derivatized samples on ice to a 4.0°C cold room. Place and secure each derivatized sample on a nutating mixer in a 4.0°C cold room as shown below.



**Figure 8:** Derivatized samples placed on the nutating mixer in the 4 degrees Celsius cold room for overnight mixing (approximately 12-16 hours). The four samples on the left were agitated on the nutating mixer for ~16 hours and the six samples on the right were just placed on the nutating mixer at the time this photograph was taken.

- 23 Allow the derivatized sample to mix undisturbed on the nutating mixer for approximately 12-16 hours to ensure complete derivatization.
- 24 Centrifuge the derivatized sample for 5 minutes at 2,000 rcf to pellet any residual debris from the derivatization reaction. This step is not essential but is best to perform this step

to reduce the potential of any debris from the derivatized sample clogging the HPLC needle, tubing, or column.

- 25 Transfer 200  $\mu$ L of supernatant from the derivatized sample to a labeled HPLC vial.
- 26 Run the derivatized sample on a Shimadzu HPLC system using the setup parameters described below along with a set of derivatized acetaldehyde standards (10 mM, 1.0 mM, 0.1 mM, and 0.01 mM).
- 26.1 **Note:** Due to an insufficient concentration of 2,4-DNPH in the derivatization reagent, the acetaldehyde present in a 100 mM standard cannot be fully derivatized and thus should not be used to create a standard curve to quantify acetaldehyde. Only the 10 mM, 1.0 mM, 0.1 mM, and 0.01 mM acetaldehyde standards should be used in the creation of a standard curve to quantify acetaldehyde.
- 27 Refer to step 13 of this protocol (under the "Preparation of Acetaldehyde Standards" section) for a description of how to convert height values (in mAu) on the HPLC system to concentration values (in mM) to quantify the amount of acetaldehyde present in each biological fermentation sample.

## HPLC System Setup and Method Parameters

- 28 Detailed below are the high-performance liquid chromatography (HPLC) system setup parameters for running the derivatized acetaldehyde samples on a Shimadzu HPLC system:
  - 28.1 **HPLC System:** Shimadzu Prominence-i LC-2030C 3D Liquid Chromatograph
  - 28.2 **Refractive Index Detector (RID) Unit:** Shimadzu RID-20A Refractive Index Detector
  - 28.3 **HPLC Column:** Shim-Pack XR ODS column (75 mm L. x 4.6 mm I.D., 2.2  $\mu$ m)
  - 28.4 **Column Temperature:** 40°C (left and right)
  - 28.5 **Tray Temperature:** 4.0°C
  - 28.6 **Mobile Phases (2 separate solutions):**  
Eluent A: Acetonitrile (100% acetonitrile)

Eluent B: Water/Tetrahydrofuran (THF) in a 8:2 ratio (ex: 800 mL milliQ water, 200 mL THF = 1.0 L solvent)

28.7 **Mobile Phase Flow Rate:** 1.0 mL/minute

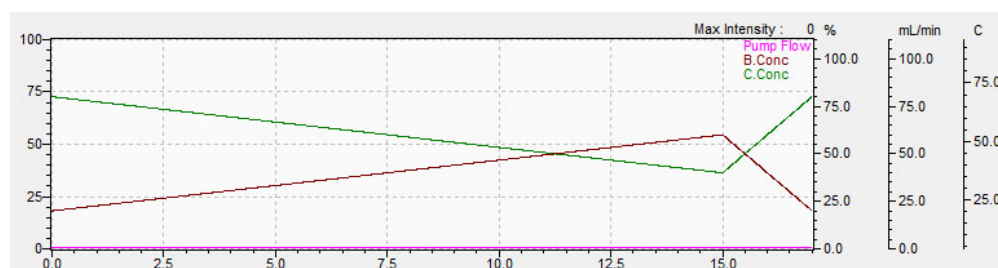
28.8 **Injection Volume:** 10.0  $\mu$ L

28.9 **Time Program (Gradient Run):** Total run time per sample = 17.0 minutes

Tables 1-2 and Figure 9 below detail the gradient run setup for Eluent A (acetonitrile) and Eluent B (Water/THF) for running derivatized acetaldehyde samples on the Shimadzu Shim-Pack XR ODS Column.

|  | Time (min) | Eluent A (Acetonitrile) Gradient (%) | Eluent B (Water/THF) Gradient (%) |
|--|------------|--------------------------------------|-----------------------------------|
|  | 0 min      | 20%                                  | 80%                               |
|  | 15 min     | 60%                                  | 40%                               |
|  | 17 min     | 20%                                  | 80%                               |

**Table 1:** Table detailing the gradient run setup for both Eluent A and Eluent B over the 17 minute run time for each sample injected onto the Shim-Pack XR ODS Column on the Shimadzu HPLC system.



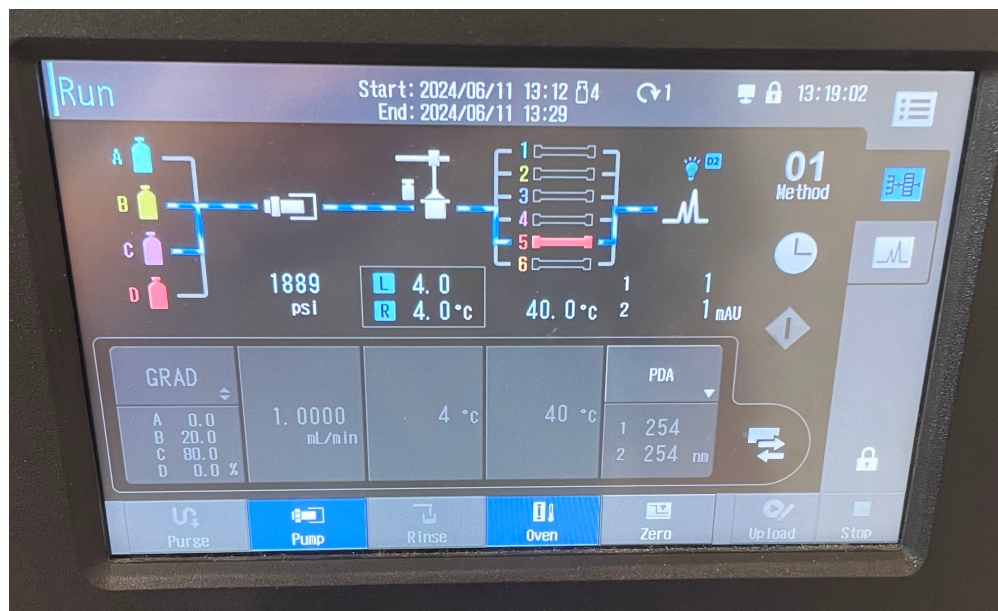
**Figure 9:** Graph of the eluent gradient setup on the Shimadzu HPLC system. \*Note that "Solvent B" in this graph corresponds to acetonitrile and "Solvent C" corresponds to Water/THF (in an 8:2 ratio) as it is setup on the Shimadzu HPLC system in the Lynd and Olson Labs at Dartmouth College.

|    | Time  | Module     | Command         | Value |
|----|-------|------------|-----------------|-------|
| 1  | 0.01  | Pumps      | Flow            | 1     |
| 2  | 0.01  | Pumps      | Solvent B Conc. | 20    |
| 3  | 0.01  | Pumps      | Solvent B Curve | 0     |
| 4  | 0.01  | Pumps      | Solvent C Conc. | 80    |
| 5  | 0.01  | Pumps      | Solvent C Curve | 0     |
| 6  | 15.00 | Pumps      | Solvent B Conc. | 60    |
| 7  | 15.00 | Pumps      | Solvent C Conc. | 40    |
| 8  | 15.01 | Pumps      | Solvent B Curve | 0     |
| 9  | 15.01 | Pumps      | Solvent C Curve | 0     |
| 10 | 17.00 | Pumps      | Solvent B Conc. | 20    |
| 11 | 17.00 | Pumps      | Solvent C Conc. | 80    |
| 12 | 17.01 | Controller | Stop            |       |

**Table 2:** Table detailing the eluent gradient run setup that corresponds to the graph in Figure 9 above on the Shimadzu HPLC system. \*Note that "Solvent B" in this table corresponds to 100% acetonitrile and "Solvent C" corresponds to Water/THF (in an 8:2 ratio) as it is setup on the Shimadzu HPLC system in the Lynd and Olson Labs at Dartmouth College.

28.10 **Detection Method:** Photodiode array (PDA) [Ultraviolet (UV)] at 254 nm

28.11 **Provided below is a photograph of the touchscreen interface on a Shimadzu Prominence-i LC-2030C 3D HPLC system while running a derivatized sample using the setup parameters described above:**



**Figure 10:** Shimadzu Prominence-i LC-2030C 3D HPLC system touchscreen interface detailing the method and setup parameters described above for quantifying acetaldehyde in derivatized samples.



## Protocol references

1. Bekers, K.M., Heijnen, J.J. & van Gulik, W.M. Determination of the in vivo NAD:NADH ratio in *Saccharomyces cerevisiae* under anaerobic conditions, using alcohol dehydrogenase as sensor reaction. *Yeast* **32**, 541-557 (2015).
2. Shimadzu. Rapid Analysis of 2,4-DNPH-Derivatized Aldehydes and Ketones Using the Prominence-i with a Shim-pack XR-ODS Column. Shimadzu Application News No. L476.  
[https://www.shimadzu.com/an/sites/shimadzu.com.an/files/pim/pim\\_document\\_file/applications/application\\_note/10903/jpl214038.pdf](https://www.shimadzu.com/an/sites/shimadzu.com.an/files/pim/pim_document_file/applications/application_note/10903/jpl214038.pdf) (2014).