29. Enzymatic Assay for PET Upcycling Enzymes

Abstract: These assays tested whether our engineered enzymes could convert TPA into activated intermediates. They directly addressed the project's upcycling goal. Positive results would provide proof-of-concept for creating new materials from PET waste. This protocol is based on the work of Kuang et al. (2007).

Materials:

- CoA-ligase (purified)
- Terephthalic acid (TPA)
- 4-Chlorobenzoic acid (4-CBA)
- p-Coumaric acid (p-CA)
- Coenzyme A (CoA) sodium salt hydrate
- Adenosine triphosphate (ATP)
- Magnesium chloride ($MgCl_2$)
- Dithiothreitol (DTT) (optional)
- Sodium pyrophosphate ($Na_4P_2O_7$) (for standard curve)
- Tris-HCl buffer (pH 7.5)
- Ammonium molybdate
- Sulfuric acid (H_2SO_4), 1 M
- 2-Mercaptoethanol
- Sodium dodecyl sulfate (SDS), 10% solution
- Deionized water
- Spectrophotometer (capable of reading 250-400 nm and 580 nm)
- Quartz cuvettes (for direct assay)
- Test tubes or microplates (for PPi assay)
- Water bath or incubator (30°C)
- Heat block (95-100°C)
- Microcentrifuge

Direct Spectrophotometric Assay

Purpose: To directly detect thioester product (CoA conjugate) based on absorbance.

Notes: Please note that before you can actually start with this protocol, there are some calculations that need to be made.

Enzyme Reaction Setup (Shared for Both Assays)

Component	***Volume to be added***	***Final Concentration***
Tris-HCl pH 7.5	835 µL (50 mM)	50 mM
$MgCl_2$	10 µL (100 mM)	1 mM
ATP	40 µL (100 mM)	4 mM
CoA	15 µL (10 mM)	0.15 mM
Substrate: - TPA (Terephthalic acid) - 4-CBA (4-Chlorobenzoic acid) - p-CA (p-Coumaric acid)	100 µL (100 mM)	10 mM
Enzyme (CoA-ligase)	Calculate based on the concentration you have of the enzyme	5 µg/reaction
Total Reaction Volume	1 mL	

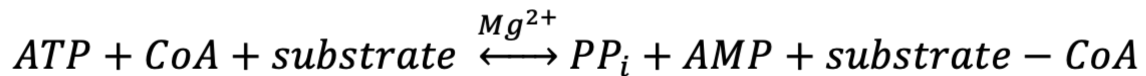
1. Mix reagents on ice. Pre-warm to 30 °C.
2. Start reaction by adding enzyme.
3. Incubate at 30 °C for 10–30 minutes.
4. After incubation, transfer to quartz cuvette or UV-compatible plate.
5. Scan absorbance from 250–400 nm to identify potential peaks from the acyl-CoA product.
    a. Known thioester peaks:
        i. 4-coumaric acid CoA ligase: ~333 nm
        ii. 4-chlorobenzoate CoA ligase: ~310–320 nm
        iii. TPA-CoA: unknown — scan broadly. Based on similar aromatic CoA thioesters, a peak may appear between 310–340 nm.
6. Compare against:
    - Reaction with no enzyme (blank)
    - Reaction with no substrate (negative control)

Optional: If you detect a clear peak, you can use that wavelength for kinetic measurements.

PPi Molybdate Assay

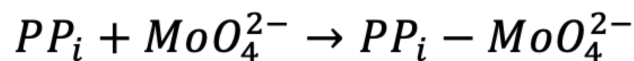
Purpose: To detect pyrophosphate (PPi) released from ATP hydrolysis as an indirect measure of CoA ligase enzyme activity.

Basis: Our CoA ligases perform the following reaction:



$$ATP + CoA + substrate \xleftrightarrow{Mg^{2+}} PP_i + AMP + substrate - CoA$$

where ATP and CoA is consumed to attach CoA to the substrate with the byproduct of pyrophosphate (PPi). In this assay, we can indirectly measure the activity of the CoA ligase enzyme by utilizing the following reaction between PPi and molybdate reagent that produces a colored compound:



$$PP_i + MoO_4^{2-} \rightarrow PP_i - MoO_4^{2-}$$

When PPi is mixed with molybdate under acidic conditions, it forms a PPi-molybdate complex.

The PPi-molybdate complex gets reduced (gains electrons) by 2-mercaptoethanol, which acts as a reducing agent. This reduction changes the molybdenum from Mo(VI) to a lower oxidation state like Mo(V) or Mo(IV). This reduction leads to the formation of blue-colored reduced molybdate complexes known as a molybdenum blue, which absorbs light at 580 nm.

The higher enzymatic activity, the more pyrophosphate should be formed, the more blue complexes should form and thus, the more light should be absorbed at 580nm.

Based on this article:
Kuang, Y., Salem, N., Wang, F., Schomisch, S. J., Chandramouli, V., & Lee, Z. (2007). A colorimetric assay method to measure acetyl-CoA synthetase activity: Application to woodchuck model of hepatitis virus-induced hepatocellular carcinoma. *Journal of Biochemical and Biophysical Methods, 70*(4), 649–655. https://doi.org/10.1016/j.jbbm.2007.02.008

Notes: Please note that before you can actually start with this protocol, there are some calculations that need to be made.

Enzyme Reaction Setup (Shared for Both Assays)

Component	***Volume to be added***	***Final Concentration***
Tris-HCl pH 7.5	835 µL (50 mM)	50 mM
$MgCl_2$	10 µL (100 mM)	1 mM
ATP	40 µL (100 mM)	4 mM
CoA	15 µL (10 mM)	0.15 mM
Substrate: - TPA (Terephthalic acid) - for testing all of our variants - 4-CBA (4-Chlorobenzoic acid) - for 1T5H variants - p-CA (p-Coumaric acid) - for 3TSY variants	100 µL (100 mM)	10 mM
Enzyme (CoA-ligase)	Calculate based on the concentration you have of the enzyme	5 µg/reaction
Total Reaction Volume	1 mL	

1. Mix reagents on ice. Pre-warm to 30 °C.
1. Start reaction by adding enzyme.
2. Incubate at 30 °C for 10–30 minutes.
3. Transfer 800 µL of the supernatant to a test tube.
4. Stop the enzyme reaction by mixing the content of the tube 1:1 with 1 M HCl and then boil the tube at 95–100 °C for 10 minutes.
5. Centrifuge to remove precipitated protein.
6. Transfer 1600 µL of the supernatant to a test tube, then follow the molybdate reagents below.

Molybdate Reagents Preparation

Needs to be prepared fresh! The total reaction volume depends on how many reactions you are going to perform. Be careful when handling the sulfuric acid. Put on PPE and work in a fume hood.

1. Prepare ascorbic acid 20 g/L in a 10 mL tube. Cover it with foil to protect it from degradation.
2. For preparation of ammonium molybdate reagent, do as follows:
    a. Pour about ~8.0 - 8.5 mL of Milli-Q (or distilled) water into a clean beaker or tube and stir.
    b. Slowly add 500 µL of 1 M H_2SO_4 to the water. Work in the fume hood.

c. While stirring, add 0.25 g ammonium molybdate to the acidified water. Cap/cover and shake or stir until fully dissolved.
d. Bring the solution up to 10.0 mL with water.
e. Label and use immediately (prepare fresh).

Preparation of ammonium molybdate reagent

Component	***Mass***	***Concentration***
Ammonium Molybdate	0.25 g (for 10 mL)	2.5%
1 M H_2SO_4	500 µL	-
Total Reaction Volume	10 mL	

1. Add 200 µL of 2.5% molybdate reagent and 200 µL of 20g/L ascorbic acid for a total volume of 2 mL.
2. Let equilibrate at 37°C for 10 min.
3. See which is the max. absorbance wavelength (previously it has been around 820 nm). Then, do single wavelength measurements.

Standard Curve

As a buffer, use TRIS-HCL pH 7.5. Use sodium pyrophosphate ($Na_4P_2O_7$) 100 µM stock to generate a standard curve:

Standard	***Final [PPi] (when acid, ascorbic acid and molybdate reagents are added)***	***Volume of 100 µM stock***	***Buffer Volume***	***Total***
Blank	0 µM	0 µL	800 µL	800 µL
Std 1	2 µM	40 µL	760 µL	800 µL
Std 2	4 µM	80 µL	720 µL	800 µL
Std 3	8 µM	160 µL	640 µL	800 µL
Std 4	12 µM	240 µL	560 µL	800 µL
Std 5	16 µM	320 µL	480 µL	800 µL
Std 6	20 µM	400 µL	400 µL	800 µL
Std 7	24 µM	480 µL	320 µL	800 µL
Std 8	28 µM	560 µL	240 µL	800 µL

Mix PPi standards with 1:1 with 1 M HCl (that is 800 µL of standard + 800 µL of HCl). Then, heat them on the heating block at 95 °C for 10 min. The reason for this is to hydrolyse pyrophosphate (PPi) into orthophosphate (Pi)

After preparing these, treat them identically to your samples (add molybdate reagent + ascorbic acid, then incubate at 37°C for 10 min).

Data Analysis

- Plot A580 vs. [PPi] for standard curve.
- Use this to calculate the amount of PPi generated in each sample.
- Normalize to time and protein amount to get specific activity.

Suggested Controls for Both Assays

Control	***Purpose***
No enzyme	Detect background hydrolysis
No substrate	Detect spontaneous ATP hydrolysis
No CoA	Check whether CoA is essential
Heat-inactivated enzyme	Negative control