WCSB, Wageningen University	IBISBA-SOP-WU19
	Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: *Enzymatic acetyl-CoA determination*

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Experimenter 1	Rita Volkers		20/3/2019

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Instruction

Enzymatic acetyl-CoA determination

1. Introduction / Purpose

Acetyl-coenzyme A synthethase (Acs, E.C.6.2.1.1) is an acetate activating enzyme widely represented in nature from bacteria to human. Its function is important for cellular catabolism, especially in order to support microbial growth at low concentrations of acetate (<10 mM) (Castano Cerezo *et al*., 2011; Castano Cerezo *et al*., 2009；Renilla *et al*., 2012). In this protocol, a continuous coupled enzymatic assay for Acs activity is described. Product formation is followed spectrophotometrically by the formation of NADH. The protocol is tailored for *E. coli*'s Acs, but it can be adapted to assay Acs in any other organism.

The acetyl-coenzyme A synthetase (Acs) assay was first described by Brown *et al*. (1977). Acs activity is measured using an enzymatic method coupled to malate dehydrogenase (Mdh) and citrate synthase (Cs):

(Acs) acetate + CoASH + ATP -> acetyl-CoA + AMP
(Cs) acetyl-CoA + oxaloacetate -> citrate + CoASH
(Mdh) L-malate + NAD+ -> oxaloacetate + NADH
Net reaction: Acetate + ATP + L-malate + NAD+ -> citrate + AMP + NADH

Under the assay conditions, Mdh and Cs activities are in excess and the rate of NADH formation is limited by Acs activity.

Keywords: Acetyl-CoA

2. Equipment and chemicals

2.1. Equipment

- Refrigerated benchtop centrifuge
- Ultrasonic homogenizer equipped with a 3 mm diameter probe
- Spectrophotometric plate reader
- Spectrophotometer

2.2. Chemicals

- Dissolve in MQ water:
- 20 mM ATP (Sigma-Aldrich: A3377)
- 2 mM Coenzyme A trilithium salt (CoASH) (Sigma-Aldrich: C3019)
- 60 mM β-nicotinamide adenine dinucleotide hydrate (NAD+) (Sigma-Aldrich: N7004)
- Dissolve in Tris-HCl buffer (see 3 Media/buffers)

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- 50 mM MgCl2 (Panreac Applichem: 131396)
- 50 mM L-malate (Sigma-Aldrich, catalog number: 02288)
- 1 M sodium acetate trihydrate (Sigma-Aldric: S8625)
- Store in at 4-6 °C and dilute in Tris-HCl buffer immediately before use (see 2.2 Media/buffers):
- 50 U/ml malate dehydrogenase (Mdh) from bovine heart (Sigma-Aldrich: M9004)
- 25 U/ml citrate synthase from porcine heart (Cs) (Sigma-Aldrich: C3260)

2.3. Other materials

Cultured cells (approx. 1010 cells) (*e.g.*, for *Escherichia coli* cells grown in glucose minimal medium, 1 ml of OD600 1 corresponds to approx. 6 x 10^8 cells)

S*pecial consumables*

- 96 well flat bottom, non-treated, non-sterile transparent plates
- Non-sterile transpartent plates
- Water-ice bath

3. Media and Buffers

- Potassium phosphate buffer 65 mM (adjust to pH 7.5 with KOH) (Sigma-Aldrich: P5379) NEEDS TO BE ICE COLD for this protocol
- 100 mM Tris-HCl buffer (adjust to pH 7.8 with KOH) (Sigma-Aldrich: T1502)

4. Procedures

Protein extraction procedure

- Harvest the cells at the phase of culture of interest, *e.g.* for *E. coli*, 50 ml of a culture in exponential growth phase (OD600=0.8) or 20 ml of a culture in stationary phase (OD600=2.0).
- Cool the cells in a water-ice bath for 1-2 min.
- Centrifuge the cells for 15 min (10,000 *x g*, 4 °C).
- Discard the supernatant.

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- Resuspend the cells in ice-cold phosphate buffer (in order to ensure proper washing of cells, use the same volume as the initial volume of sample).

- Centrifuge the cells for 15 min (10,000 *x g*, 4 °C). Discard the supernatant.

- Resuspend the cells in ice-cold phosphate buffer. Use an appropriate volume of buffer, calculated according to the following equation; this will result in a cell suspension of OD600 of:

  50ml (phosphate buffer)=(OD600 x Vol (ml culture volume))/50

- Where OD600 is considered as readout of the concentration of cells in the sample (Castano-Cerezo *et al.*, 2011).

- Disrupt the cells using an ultrasonic homogenizer as follows. Transfer the cell suspension to a 1.5 ml conical tube and place it in an ice bucket. Subject the cell suspension to 3 sonication pulses (20 sec each, 21% amplitude, 3-mm diameter probe, 50% power input). Respect 60 sec intervals between sonication cycles in order to avoid over-heating of samples.

- Centrifuge the disrupted cells for 30 min (20,000 *x g*, 4 °C).

- Collect the supernatant (cell-free extract) and transfer it to a clean 1.5 ml conical tube. Inspect the cell-free extract for suspended particles. If necessary, centrifuge again.

- Use the cell- free extract immediately for acetyl-CoA synthetase assay or store the supernatant at -70 °C for long term use.

- Acetyl-coenzyme A synthethase assay

- For proper determination of enzyme activity, the optimal dilution of the protein extract has to be determined for each sample, since the activity of extracted enzyme is influenced by the physiological state and the extraction yield of the cells.

- Thaw the protein extracts and reagent stocks on ice. Dilute the malate dehydrogenase (Mdh) and citrate synthase (Cs) enzyme stocks as explained in the Materials section and keep them on ice.

- Connect the plate reader and allow it to reach 37 °C.

- Prepare a "Master Mix" according to the number of samples to be analyzed. The "Master Mix" should contain all the reaction components except for sodium acetate (the substrate of Acs) and the protein extract. Remember to consider sample triplicates. Keep the "Master Mix" on ice.

- "Master Mix" per reaction:
  100 mM Tris-HCl buffer (pH 7.8): 70 µl
  50 mM L-malate : 10 µl
  20 mM ATP : 10 µl
  50 mM MgCl2 : 10 µl
  2 mM CoASH : 10 µl
  60 mM NAD+ : 10 µl
  50 U/ml Mdh : 10 µl
  25 U/ml Cs : 10 µl

- Prepare several dilutions of the protein extract in phosphate buffer. Choose dilutions in a wide enough range (*e.g.*: 1:1, 1:10, 1:100 and 1:1,000).

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- In a 96 well flat bottom, transparent plate, add 140 µl of the Master Mix to each well.

- Add 40 µl of each of the protein dilutions. Pipet up and down in order to mix the reaction components properly. Tip: Avoid the formation of air bubbles.

- Start the reaction by adding 20 ul of 1 M sodium acetate into the sample and mix well by pipetting up and down.

- Incubate at 37 °C in the plate reader and record the continuous increase in absorbance at 340 nm (due to the reduction of NAD+) for 5-10 min. Recording should start immediately after starting the reaction. Adjust the reader to get an absorbance value each 10-20 sec.

- Calculate the maximum slope in the OD over time plot (ΔOD/min) for each dilution of the protein extract. For subsequent measurements, select the 2-3 dilutions of the extract to yield a ΔOD/min=10-200. If necessary, assay other dilutions (the typical optimal dilution for Acs activity in cell extracts of glucose-limited stationary phase cultures of *E. coli* extracts with a protein concentration of approx. 15 mg/ml is 1:20).

- Repeat the assay with the dilutions selected. Perform the assay at least in triplicate. To ensure proper determination of enzyme activity in the extract, ΔOD/min should be proportional to protein content.

- Calculate the Enzyme Activity Units for the protein extract assayed using the following equation:

- Acs Activity (U/ml) = (ΔOD (/min)*Vr*Df) / (ε *l*Ve)

- Where:
  ΔOD (/min) = maximum slope in the OD vs. t plot (/min)
  Vr = reaction volume (µl)
  Df = dilution factor
  ε NADH 340 nm = 6.22 ml*/µmol*/cm
  l = optical path length (cm)[a]
  Ve = protein extract volume (µl)[b]

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  [a]) To determine the optical path length, refer to the specifications of your 96 well plates to get the diameter of the well. Calculate the optical path length from the total liquid volume used in the assay and the diameter of the well.
  [b]) This volume refers to the volume of diluted extract added to the assay.

- Determine the protein concentration of the extracts assayed [*e.g*. using the Lowry (Hartree, 1972) or the Bicinchoninic Acid method (Smith *et al.,* 1985)].

- Determine the specific Acs activity for each extract using the following equation:

  Acs Specific Activity (U/mg) = Acs Activity (U/ml) / Protein concentration (mg/ml)

5. Remarks / troubleshooting

- This is a complex protocol. Read it very carefully before you start!
- When pipetting up and down to mix the contents of the 96-wells plate: avoid the formation of air bubbles.
- Perform the assay at least in triplicate!

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6. Referencs

This protocol was first described in and adapted from Brown *et al.* (1977) and previously used in Castano-Cerezo *et al.*(2009) and Castano-Cerezo *et al.* (2011).

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