NAD ASSAY

REAGENTS

1: GLYCYL-Glycine BUFFER (x 250 ml) at pH 7.4

0.065 M glycyl-glycine in distilled water
3.05 g Niacinamide
6.05 ml 95% ethanol

2: ALCOHIOL DEHYDROGENASE (ADH)

from a stock solution of 29.4 mg/ml pick up 20 μl and dilute in 1 ml of phosphate buffer (10 mM at pH 7). **20 μl/ml**

3: MYF

2.5 mg/ml distilled water

4: PHENAZINE METOSULFATE (PMS)

4 rng/ml distiiled water

METHOD

1: SAMPLES PREPARATION

In 12 well plates:

Remove supernatant off cells
Add 250μl Perchloric add 0.5 N and scrape the cells
Transfer the cells to eppendorf tubes
Add 70 μl of KOH 3M to neutralize
Centrifuge for 2 minutes at 40 °C

2: ASSAY

- In 96 well plates add for each well:

190 μl Glycyl-glycine buffer
10 μ1 ADH
10 μl MTT
20 μL PMS

- Incubate the plate for 10 minutes at 37C in the plate reader
- Add 20 μl of sample to each wells with the above reagents
- Make the blank with all the above reagents but <u>without</u> the samples
- Make the standard with all the above reagents with 20 μl of NAD 1μM
- Read al 560 nm at 0, 10 and 20 minutes at 37 °C.

3: CALCULATION

- Subtract blank from the OD values of samples (or read subtacting the blank)
- Plot OD values on y axis and time on x axis and calculate the simple slope
- Divide the obtained slope by the standard slope
- Data are expressed as nmol/ml or n cells of NAD

CELL CULTURE PREPARATION FOR ATP
(6-well and 12-well plate prep.)

CELL SCRAPING

1. After removal of the cell culture plates from the incubator, keep on ice until each well is ready for scraping.
2. Aspirate 50u1 media from each well for nitrate and place in a well of a 96-well plate. (store at -20 C until nitrate analysis)
NOTE: omit step #2 if nitrate determination is not needed.
3. Aspirate remaining media from wells without touching cell monolayer.
4a. For 6-well plates: Immediately add 1.0cc of iced perchloric acid (0.6N) to each well.
4b. For 12-well plates: Immediately add 0.5cc of iced perchloric acid (0.6N) to each well.
5. With a cell scraper, scrape cells from each well, gently mixing with the perchloric acid.
6. Transfer cells into labelled eppendorf tubes and place on ice.
7. Vortex each epp. tube for l0sec. at speed 5.

CELL SONICATION

8. Using Virtis membrane disruptor, sonicate each epp. tube containing the cell extract for 10 sec at setting #5. (position the epp. tube in an ice bath while sonicating)
9. Leave epp. tubes on ice for 60min.

NEUTRALIZATION

10a. Vortex epp tube 5sec
10bi. For 6-well plates: Immediately add 450u1 of "ICED" K2HPO4 (1.0M pH 12)
10b2. For 12-well plates: Immediately add 225u1 of "ICED" K2HPO4 (1.0M pH 12)
l0c. Vortex again for 5sec
11. Centrifuge at 10,000g for l0 min at 4 C

FILTRATION

(Using 3cc syringes, l9ga X 1.5inch needles, gelman acrodisc or equiv. 0.2u syringe filters.)
12. Aspirate supernate from the pellet with the 3cc syringe and needle.
13. Remove needle and attach syringe filter to the syringe.
14. Filter sample into a labelled micro-vial.
15. Immediately drop vial into container of liquid nitrogen to freeze instantly.
16. Store at -70 C until ATP analysis

DIGESTION OF CELL PELLET

17. Invert the pellet to allow to dry at room temp.
18. Add 1.Occ of NaOH (1.ON) to pellet and vortex until pellet is mixed.
19. Store at room temp. until protein is assayed.

PROTEIN (Pellet) ANALYSIS

1. Centrifuge the protein digest for l0 minute at 4,000RPM at room temp.
2. Set-up 1 2X75mm test tubes for the protein determination.
3. In each tube, add 800u1 dist. H_20
4. Add 200u1 Bio-Rad dye reagent and vortex.
5. Next add protein digest sample: add 50ul.
6. Immediately VORTEX again right after adding the protein sample!!
7. Transfer 200u1 of each test tube sample into 96 well plate.

8. Prepare SpectraMax for protein analysis:
a) Double click MacHD
b) Click on Open Settings
c) Click on Settings Protein/Bio-Rad
d) Go to Template
e) Paint the plate according to where your sample wells are located.
f) Click on Group and go to Unknowns and Click O.K.
h) Open drawer on plate reader SpectraMax machine and put 96 well plate in position.
i) Go back to computer and click on READ!!!
j) Go to file and Print Plate 1, print 2 copies for Alvin.