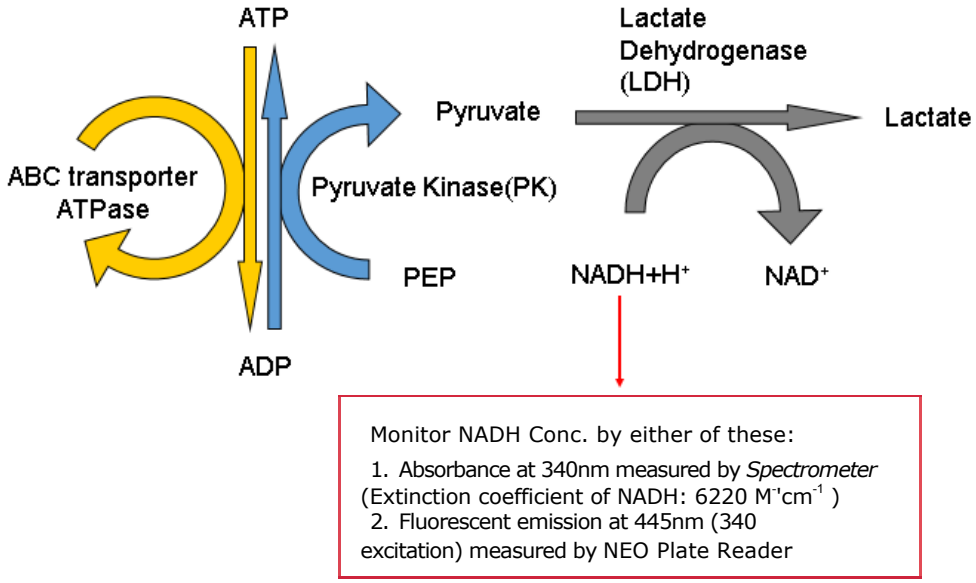
ATP/NADH Coupled ATPase Assay Protocol with Synergy Neo Multi-Mode Reader



Stock solution to make:

1M HEPES, pH 8.0
50mM NADH (Roche, 4°C. Freshly made or store stock at -80°C)
0.2M ATP (Sigma, -20°C. pH to 7.0 with KOH and store at -80°C)

5mL Reaction Buffer:	**Final Conc.**
250uL 1M HEPES, pH 8.0	50mM
30uL Pyruvate Kinase (PK), 10mg/mL stock (Roche, 4°C)	60mg/mL
32uL Lactate Dehydrogenase (LDH), 5mg/mL stock (Roche, 4°C)	32mg/mL
9.3mg phosphoenolpyruvate (PEP), powder, (Roche, 4°C)	9mM
15uL (30uL) 50mM NADH	0.15mM (0.30 mM)
(50uL 0.1M $MgCl_2$, added according to your needs)`	(1mM)
4.658 ddH_2O	

OPTIONAL: DTT or buffers at different pH

To Measure ATPase activities with NEO Plate Reader:

1) Generate a standard curve of NADH fluorescence vs. NADH concentration at time 0 with only NADH in buffer. (Make sure NADH conc. used in the reaction buffer falls in the linear range.)
2) To measure ATPase activities:
    a. Sample: mix 0.5mL reaction with desired amount of your ATPase (For PCAT1, 0.066mg/m1 protein was used in the reaction to measure basal activities). Control/background: mix 0.5mL reaction with H_2O instead of ATPase.
3) Aliquot 30uL reaction mix or control to each well. (Make sure to measure enough duplicates to eliminate errors due to pipetting or air bubbles.)
4) Add 1-2 uL of desired amount of ATP to each well by 384 well multi-channel pipette.
5) Run ATPase coupled protocol saved in the machine with desired temperature, duration, and frequency of measurement. (Get training for the plate reader from another lab member).

6) Export the measurements and plot both control and real measurement in excel.
7) Calculate background NADH oxidation rate with the control by calculated the slope of fluorescent decrease over time.
8) Calculate ATPase rate from the slope of fluorescent decrease over time and substrate away the background NADH oxidation rate.
9) Convert the ATPase rate to nmol/mg/min:
    a. Calculate the rate NADH disappearance (mM/min) from the standard curved measured at 1).
    b. Convert mM/min to umol/mL/min
    c. NADH disappearance= ATPase rate, so divide the rate by amount of ATPase used (mg/mL) to get the rate with the unit of nmol/mg/min

10) If ATPase in the proteoliposomes were used in the measurements, divide the rate by the ratio of ATPase facing outside if it's known. Otherwise multiple the rate by 2 fold assuming half of the ATPases are inside, and half are outside of the liposome.