Shanshuang’s protocols

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- Preparation of soybean PC
  - Soybean PC is in powder stored at 4°C
  - Step 1: Dissolve 100 mg soybean PC at 50 mg/ml concentration in 2ml of buffer, typically 50 mM Tris pH 7.5. Vortex until no clump is visible.
  - Step 2: Transfer into a glass tube (with cap), purge the liquid surface with a flow of nitrogen or argon for 10 min. Close the cap.
  - Step 3: Sonicate the lipid to clarity with a sonication water bath.
- Tip: the choice of buffer should contain minimum salt; otherwise lipids after sonication will not be as clear and transparent. Some NaCl/KCl (less than 500mM) can be tolerated.

- Preparation of Synthetic lipids
  - Synthetic lipids, such as POPC, POPE, POPG, are often stored at -20°C in chloroform at 25mg/ml.
  - Step 1: Using a glass transfer pipet, take desired amount of lipids in a glass tube. Mix different lipids as necessary in desired ratio. (e.g. POPE:POPG=3:1 w/w is a good mimic of bacterial lipids.)
  - Step 2: Dry out chloroform with an argon flow.
  - Step 3: Re-dissolve lipid in pentane, and dry out pentane with an argon flow.
  - Step 4: Cap the tube loosely, and leave tube in a vacuum chamber for 1 hour or longer, even overnight.
  - Step 5: Re-suspend lipid to desired concentration in a buffer, typically 50 mM Tris pH 7.5. Vortex until no clump is visible.
  - Step 6: Purge the liquid surface with a flow of nitrogen or argon for 10 min. Close the cap.
  - Step 7: Sonicate the lipid to clarity with a sonication water bath.
- Tips: same as last page

- Expression of Membrane Scaffold Protein (MSP)
  - Step 1: Transform pMSP1E3D1(Kan^r) into BL21(DE3)
  - Step 2: Inoculate a single colony into 50ml LB supplemented with 30mg/L Kanamycin. Shake at 37°C for 5~6 hours until OD_{600} reaches ~0.6
  - Step 3: Inoculate all 50ml culture into 0.5L TB supplemented with 30mg/L Kanamycin. Shake at 37°C for 3~4 hours until OD_{600} reaches ~0.6
  - Step 4: Induce with 1mM IPTG. Keep shaking at 37°C for 3 hours
  - Step 5: Spin down cells at 4000 rpm with low-speed (J6-MI) centrifuge for 15 min at 4°C.

- Purification of Membrane Scaffold Protein
  - Step 0: Prepare Buffer A (40 mM Tris, pH 8.0, 300 mM NaCl) and chill it at 4°C. All following steps should be conducted at 4°C.
  - Step 1: Resuspend cells from 0.5 L culture in ~30ml of Buffer A supplemented with DNAse and 1% Triton X-100.
  - Step 2: Break cells by two passages through high-pressure microfluidizer.
  - Step 3: Spin down to remove cell debris at 70,000 g for 30 min.
  - Step 4: Incubate supernatant with 5ml Ni beads pre-equilibrated with Buffer A + 1% Triton X-100 in a 50 ml corning tube for 1 hour with gentle rocking.
  - Step 5: Load the mixture on a disposable column.
  - Step 6: Wash with
    - 1) 50 ml of Buffer A + 1% Triton X-100
    - 2) 25 ml of Buffer A + 50 mM Na Cholate + 20 mM imidazole
    - 3) 35 ml of Buffer A + 50 mM imidazole
  - Step 7: Elute MSP with 40 ml of Buffer A + 400 mM imidazole.
  - Step 8 (Skip if intend to remove the His-tag): Dialyze the elution against 1L of Buffer A to remove imidazole.

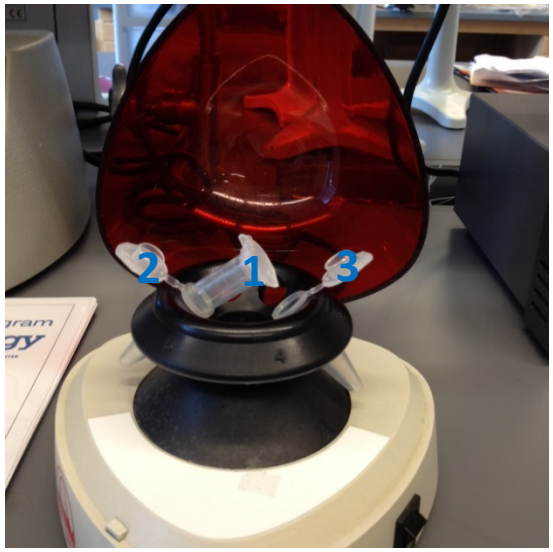
- Removal of His-tag from MSP (optional)
  - Step 9: Add TEV to MSP at 1:40 (w/w) ratio; add β-ME to 5 mM. Incubate for 3 hours to overnight.
  - Step 10: Dialyze the elution against 1L of Buffer A to remove imidazole.
  - Step 11: Remove TEV and uncleaved MSP by loading the mixture to Ni column pre-equilibrated with Buffer A + 25 mM imidazole.
  - Step 12: Concentrate MSP with 30 kDa cutoff concentrator to desired concentration.
  - Step 13: Aliquot and flash-freeze in liquid nitrogen.

- Nanodisc Assembly
  - Example: wt MalFGK$_2$ with Soybean PC
    - MSP:protein = 5 (molar ratio);
    - Lipids:MSP = ~150 (molar ratio);
    - 500 µl final volume
  - Step 1: Add components in the following order.

order	reagent	Stock conc	Volume	Final conc
1	Tris, pH7.5	50 mM	34 µl	
2	Na Cholate	500 mM	25 µl	25 mM
3	Soybean PC*	50 mg/ml	60 µl	6 mg/ml
4	wtMalFGK$_2$	20 mg/ml = 116 µM	44 µl	10 µM
5	MSP	2.2 mg/ml = 74 µM	337 µl	50 µM

    - *: prepared as described in page 3.
  - Step 2: Incubate the mixture at room temperature with gentle rocking for 45~60 min. (to be continued…)

- Nanodisc Assembly (continued)
  - Step 3: While waiting for Step 2, prepare ~0.5 ml Bio-Beads by a methanol wash, followed by more than 5 times of water wash, finished by a reconstitution buffer wash. (to be continued…)
    - Tip for wash: transfer more than 0.5 ml BioBeads into a 15ml tube; pierce a hole in the V-bottom of the tube; add methanol/water/ buffer, shake well, and dry the beads by vacuum. See picture on the left. For further drying, re-suspend the beads with ~ 1ml of buffer; transfer the slush into a 1.7 ml tube with bottom pierced, set up as shown in the picture on the right and briefly spin. (1: Bio-Beads Slush; 2: waste; 3: balance)



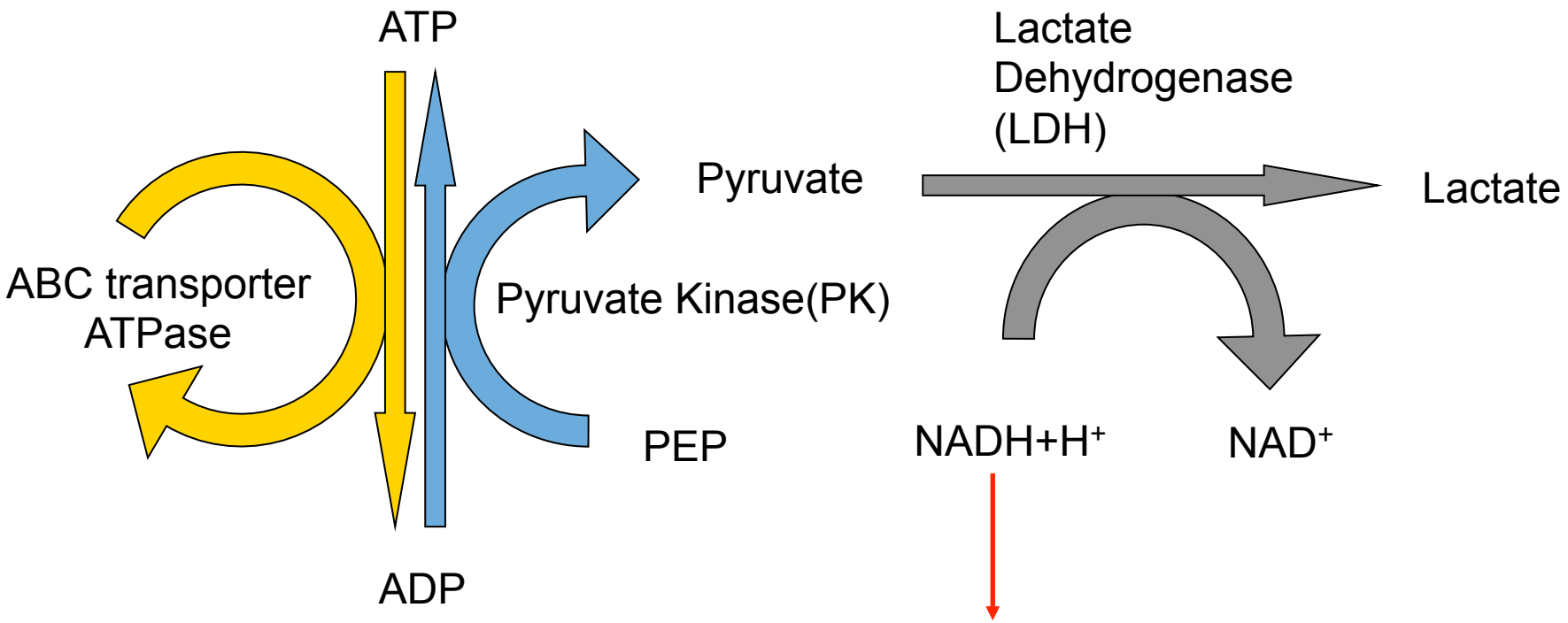
- Nanodisc Assembly (continued)
  - Step 4: Transfer ~0.5ml of dried Bio-Beads to an intact 1.7 ml tube, and add the entire mixture (500 µl) from Step 2. Incubate at room temperature with gentle rocking for 3 hours. Nanodisc self-assembly should occur upon removal of detergent by Bio-Beads.
    - IMPORTANT: room temperature of 22~25 °C is critical for self-assembly of nanodisc. Lower temperature is not good for nanodisc formation with the maltose transporter.
  - Step 5: To remove Bio-Beads, pierce a hole in the bottom of the tube containing nanodisc/Bio-beads slush, briefly spin using the set up as shown in the picture on last page (right). 1: tube containing slush; 2: fresh tube to collect sample; 3: balance.
  - Step 6: Collect nanodisc sample from tube 2 and remove residue Bio-Beads with a pipet.

- Nanodisc Assembly (continued)
  - Step 7 (optional): to remove transporter-free nanodiscs, use His-tag free MSP if transporter is His-tagged. Nanodisc sample from last step can be further purified using the affinity tag on the transporter. Avoid usage of detergent during this step of purification.

- Proteoliposome Reconstitution – Fast dilution
  - Positive control: wt $MalFGK_2$ with Soybean PC
  - Limitation: Protein has to tolerate β-OG.
  - Step 1: Prepare lipids as described in Page 3.
  - Step 2: Take 200 µl of 50mg/ml lipids, add 20 µl of 15% β-OG. Incubated at room temperature for 10 min.
  - Step 3: Concentrate $MalFGK_2$ to greater than 3.3 mg/ml, add 100 µg protein to above mixture. Incubate on ice for 10 min or more.
  - Step 4: Dilute the mixture from Step 3 by greater than 25 fold, so that the concentration of β-OG falls well below its CMC, and in turn triggers proteoliposome reconstitution. Alternatively, dilute 1/20 of the mixture (5µg of transporter) from Step 3 directly into ATPase activity assay for ATPase determination.
  - Step 5 (optional): To concentrate proteoliposome from last step, spin down at 100,000g at 4°C for 40 min, discard the supernatant and re-suspend the pellet.

- Proteoliposome Reconstitution – Dialysis
  - Step 0: Prepare a "Reconstitution buffer", e.g. 20 mM HEPES, pH7.4, 150-450 mM KCl; a DM stock at 500 mM;
  - Step 1: Prepare 20mg/ml lipids as described in Page 4 using Reconstitution buffer.
  - Step 2: Take desired amount of lipids, add DM, 10 µl a time, to a final concentration of 10mM.
  - Step 3: Put argon on top of the lipids, and incubate at room temperature for 1-2 hours with gentle rocking. Sonicate again to clarity if necessary.
  - Step 4: Prepare protein dilutions such that mixing protein and lipids at 1:1 volume ratio gives the desired protein lipid ratio. (For example, mixing equal volume of protein at 2mg/ml and lipid at 20mg/ml yields 1:10 w/w ratio).
  - Step 5: Mix equal volume of protein and lipid.
  - Step 6: Add additional DM to raise its concentration from 5 mM to 17.5 mM.
  - Step 7: Put argon on top of the lipids, and incubate at room temperature for 2 hours with gentle rocking.
  - Step 8: Using 10,000 MWCO, Dialyze the mixture against Reconstitution buffer at 4 °C for 4-5 days. Change buffer every 12 hour.
  - Step 9*: Treat Bio-Beads as described in Step 4, Page 10, and add a small amount to proteoliposome to remove any residue detergent.
    - *: for maltose transporter with PE:PG (3:1 w/w) at 1:10 protein: lipid w/w ratio, this step can be omitted.

- ATPase activity assay – Enzymatically coupled assay



Extinction coefficient at 340nm: 6220 $M^{-1}cm^{-1}$

- Prepare:
  - ATPase Rxn Mix:
    - 50 mM HEPES(KOH), pH 8.0;
    - 10 mM $MgCl_2$;
    - 60 µg/ml PK;
    - 32 µg/ml LDH;
    - 4 mM PEP;
    - 0.3 mM NADH;
  - 200 mM ATP
    - Dissolve ATP powder in 0.1 M NaOH, and add additional NaOH until pH is ~7.5 as indicated by pH paper.
- To measure activity, add protein to ATPase Rxn Mix, supplement necessary ligand(s) including ATP, and monitor decrease in OD_{340nm} with a spectrophotometer in a kinetic mode.
  - Negative control: Measure ATPase Rxn Mix supplemented with 2 mM ATP without addition of any ATPase. Make sure the slope is negligible to expected transporter ATPase activity.
  - Positive control (recommened): Measure MBP stimulated wtMalFGK$_2$ ATPase activity.
    - For proteoliposome/nanodisc sample, expect lower than 30 nmol/mg/min basal activity and 1000-5000 nmol/mg/min stimulated activity (15 µM MBP/maltose);
    - For sample in detergent, expect 100-500 nmol/mg/min basal activity 4-5 fold of stimulation (15 µM MBP/maltose).

- Read this page if you wish to understand how measured OD340 is converted to ATPase activity.
  - To convert the slope (a.u./s) of the data into ATPase activity (nmol ATP hydrolysis/(mg ATPase)/min):
    - Let Slope = A (a.u./s), ATPase concentration= E (mg/ml)
    - These two variables will be canceled during calculation: reaction volume = V (ul), reaction duration = T (min)
    - Cuvette length = 1cm
  - Disappearance of NADH (nmol) = V(ul)*A(a.u./s)*T(min)/[6220(L/mol)(1/cm)*1cm]
  - ATPase: Disappearance of NADH/T/(E*V) = A(au/s)/[(6220 L/mol)*(E mg/ml)]
    - That is, if you use 5ug of protein for a 311ul reaction (or 1.6 ug protein for 100 ul reaction) and get a reading of A (a.u./s), your ATPase activity is -600000 A nmol/mg/min

- ATPase activity assay: Molybdate Blue assay
  - Direct measurement of inorganic phosphate release.
  - Step 1: Prepare stock solutions:
    - 10% SDS, use BioUltra grade to minimize background phosphate.
    - 167 mM Ammonium Molybdate
    - 100 mM Tin Chloride ($SnCl_2$) in 1 N (0.5 M) H_2SO_4
    - 10N (5M) H_2SO_4 (to make 5 ml, add 1.38 ml of 98% H_2SO_4 to water)
  - Step 2: Make a "quenching mix" from above stocks
    - Take 14.5 ml SDS, add 3.5 ml H_2SO_4 , mix well, add 1 ml Ammonium Molybdate.
    - Aliquot as needed. Typically, 190 µl each.
  - Step 3: ATPase reaction: mix ATPase with desired ligands, add ATP last to initiate ATPase reaction and start timing.
  - Step 4: At different desired time points, take 150 µl reaction and mix into quenching mix to stop the reaction.
  - Step 5: Add 10 µl $SnCl_2$ to develop color. Mix well and incubate for 5 min.
  - Step 6: Take 300 µl of the above mixture, measure OD_{595nm}.
    - For quantification purpose, a standard curve will be necessary. Also, ATP hydrolysis should be restricted to be within 10% to measure initial rates.
    - Volumes above are for 96-well plate format; for individual 1ml cuvettes, triple all volumes.

- Appendix:

Protein constructs	Molecular Weight (kDa)	Extinction coefficient at 280 nm ($M^{-1}cm^{-1}$)
wtMalFGK$_2$	171	193
MalF(ΔTM1)GK$_2$	167	174
MalF(ΔP2)GK$_2$	153	165
MalF(ΔTM1&ΔP2) GK$_2$	149	146
Cross-linked MalEFGK$_2$	212	260
wtEIIA	18	0
wtMBP	41	66
His-MSP	32.6	29.9
MSP	30.0	26.0