5.36 Biochemistry Laboratory
Spring 2009

SESSION 12 (lab open 1-4 pm)

Your TA will assign you one or several of the following buffers, all of which will be used for the coupled phosphorylation assay in Sessions 13 and 14.

- **10 x Kinase Buffer**: 1 M Tris base (FW 121.14) pH 7.5, 100 mM $MgCl_2$.This should be prepared in several steps:
    1. First prepare 1 L of a 1M solution of Tris: In a 1 L bottle, combine _____ g Tris, and 0.8 L of room temperature DI water. Adjust the pH to 7.5 with HCl (it is recommended to start with concentrated HCl and then switch to 1.0 M HCl as you get close to the desired pH.). Add water to a final volume of 1 L.
    2. Next prepare 10 mL of an approximately 3 M solution of $MgCl_2$-$6H_2O$ (FW 203.31) in 0.1 M HCl. Record the actual concentration of your solution. The 0.1 M HCl keeps the solution acidic to prevent the $MgCl_2$ from crashing out of solution during storage.
    3. Prepare 10 mL of the 10 x kinase buffer (to avoid $MgCl_2$ crashing out at neutral pH, do not store it for more than a few weeks). For example, if the $MgCl_2$ solution was 2.66 M, you should add 385 uL of 2.66 M $MgCl_2$ to 9.615 mL of the 1 M Tris buffer, pH 7.5. The Tris concentration will be only negligibly less than 1 M.

- **100 mM ATP, pH 7.5** (ε_{259} = 15400 $M^{-1}cm^{-1}$). ATP FW = 551.14 (anhydrous). To prepare 9 ml of a 100 mM ATP solution, add _____ g of ATP to 7 mL water. Adjust the pH of the solution to 7.5 using 1 M NaOH (approximately 1.3 mL will be needed for the pH adjustment). Be careful NOT to overshoot the pH! If the solution becomes too basic, the ATP will hydrolyze and will be useless in the upcoming kinase assay. If you overshoot the pH, you should discard your solution and start over.

  After reaching a pH of 7.5, add water to a final volume of 9 mL. Determine the exact pH of the ATP solution by absorption spectroscopy. The apparent weight of the ATP is likely to include some water, which results in a lower than expected concentration. Record the Abs at 259 for a 1/4000 dilution of the ATP solution and calculate the actual ATP concentration using Beer's Law (Abs = εcl). Aliquot the ATP solution into 100-uL samples in 0.5 mL-eppendorf tubes and store the labeled-aliqots at -20 ºC.

- **35 mM PEP**. FW = 208.04. Add 100 mg of PEP to 13.7 mL of water. Store the solution in 200-µL aliquots in the -20 °C freezer. **(provided by TA)**

- **12 mM NADH**. (FW of the hydrate: 709.40). Since NADH easily takes on water weight, attempt a 14 mM solution by adding "10 mg"/mL of NADH. In a 15-mL conical tube, add "5 mg" of NADH to 0.5 mL of water. Calculate the actual concentration (which will likely be close to 12 mM) by measuring the absorption at 340 (ε_{340} = 6220 $M^{-1}cm^{-1}$). The pH of NADH solution does not need to be adjusted. Store labeled aliquots at -20 °C**. (provided by TA)**

- **20 mM stock of the substrate peptide**, Ac-EAIYAAPFAKKK-NH2. (MW 1377.6584). For a 900 µL solution of peptide (final volume), add 25 mg (18 µmol) of peptide to a 15-mL conical tube. Add 700 µL of 100 mM Tris, pH 7.5 or water to the lyophilized peptide. The peptide solution will initially be very acidic due to TFA contamination from peptide HPLC purification. To neutralize the solution, add 1 M NaOH until the pH is approximately 7.5. For one previous preparation of this peptide, 9 uL of 1 M NaOH was required for neutralization, but this will differ with every batch of peptide, depending on how much residual TFA is with the peptide. After neutralization, add 100 mM Tris buffer or water to give a final volume of 900 µL. The peptide should be labeled, dated, and stored at -20 ºC in 50-µL aliquots. **(provided by TA)**

Once the buffers above are made, all groups should prepare their own 1x assay buffer to use in Sessions 13 and 14:

Preparation of 2 mL* of 1 x assay buffer

In order to save time and allow you to easily and reproducibly repeat the coupled phosphorylation assay, you should prepare a 1x assay buffer that contains all of the reagents in the kinase assay *except* for the kinase, the substrate peptide, and the PK/LDH (coupling enzymes). Once you make up this buffer, you should store it at -20 °C. It is ok to freeze/thaw the buffer and use it as needed.

** 2 mL refers to the final volume after the addition of the kinase, peptide, and PK/LDH. The volume of the assay buffer alone will be 1.44 mL.*

For 2 mL* of 1x assay buffer, combine the following in a 2 mL eppendorf tube:

- _____ µL of 10x kinase buffer
- _____ µL of 35 mM PEP for a final concentration of 1 mM
- _____ µL of _____ mM ATP for a final concentration of 2 mM
- _____ µL of ____ mM NADH for a final concentration of 0.11 mg/mL or 155 µM. (Make sure the frozen stock that you use is recent.)
- _____ of distilled water to give a "final" volume of 1440 µL
  (This leaves 560 µL of spare volume for the enzymes and peptide.)

Store your 1x assay buffer in 400-µL aliquots in the -20 C freezer. Label your tubes well so the assay buffer is not confused with the 10x kinase buffer!