5.36 Biochemistry Laboratory
Spring 2009

5.36 Lecture Summary #6 March 17, 2009

Upcoming CI-M due date: The final draft of your mini-review is due at **noon** on Tuesday, March 31st. Reviews should be submitted on the MIT class website. *Please note that there will be a 5-point penalty for late submissions. An additional 5 points will be deducted for each subsequent day that the assignment is late.*

Next Laboratory Sessions: #12 and 13

Topics: **Detecting Kinase Activity in vitro and in vivo**

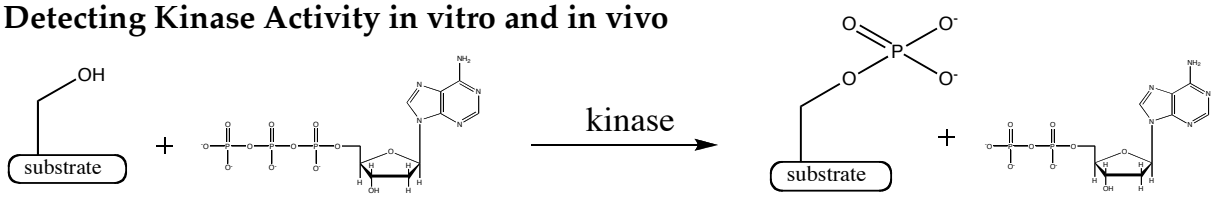
I. In vitro activity assays (for purified kinases)
- **A.** ^{32}P incorporation assay
- **B.** Gel analysis with a phospho-specific antibody
- **C.** Kinase coupled-activity assay (lab sessions 13 and 14)

II. Fluorescent peptide-based probes
- **A.** Environment-sensitive fluorescent probes
- **B.** Chelation-enhanced fluorescent probes

III. In-vivo protein-based probes (ie. FRET)

IV. *Laboratory report guidelines*

Detecting Kinase Activity in vitro and in vivo



ATP ADP

Why are scientists interested in detecting kinase activity?

- Enables monitoring of kinase activity and inhibition
- Used for screening of _____
- Facilitates the unraveling of complex cell-signalling pathways implicated in processes ranging from cell cycle regulation to cellular _____ (including tumor metastasis).

Enzyme activity assays detect either product formation or starting material consumption.

This is straightforward if a reactant or product has distinct spectroscopic properties (for example, a high extinction coefficient only in the product form.)

For kinases this means

- Detection of phosphorylated _____ (product) *or*
- Detection of _____ (product) formation or _____ (starting material) consumption

However, phosphorylation is spectroscopically _____. Neither the formation of phosphorylated product nor the conversion of ATP to ADP can be monitored *directly* using spectroscopy.

The products appear _____ to the starting materials due to identical absorption features and extinction coefficients.

Scientists have overcome this challenge creating a variety of assays.

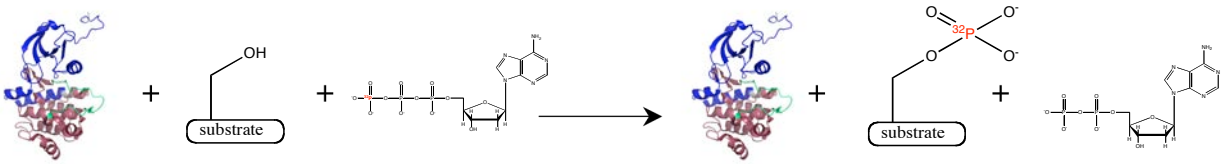
Kinase assays: in buffer, in cell lysate, in vivo
Depending on your application, you should consider whether you need an assay that can be performed only with purified enzyme, in a cell lysate mixture, or in vivo.

Some other considerations may include a protocol that is:

- Easy and safe to use
- _____ versus specific
- _____ (versus an end-point assay). Continuous assays simplify kinetic studies.
- A turn-on versus a turn-off assay (some researchers prefer the generation of a positive signal rather than a decrease in signal)
- Amenable to high-throughput applications
- Sensitive to low levels of kinase activity

I. IN VITRO ACTIVITY ASSAYS (FOR PURIFIED KINASES)

A) ^{32}P incorporation assay : detection of a radiolabeled, phosphorylated substrate



kinase substrate [^{32}P]ATP kinase ^{32}P-substrate ADP

(plus unreacted [^{32}P]ATP and substrate)

The kinase of interest is incubated with a substrate peptide or protein, radiolabeled [γ-_____]_____ and MgCl.

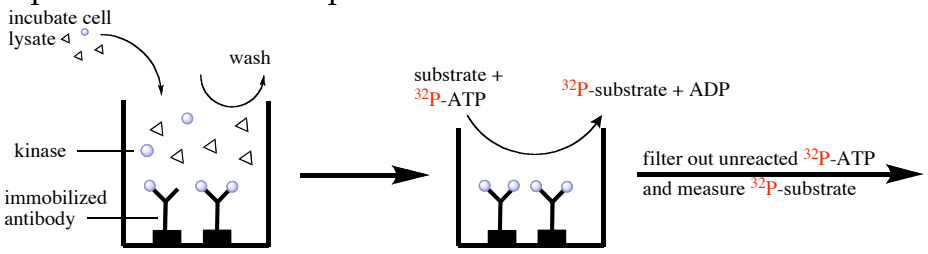
Following _____ *of the unreacted [γ-^{32}P]ATP*, the incorporation of ^{32}P into the substrate is quantified on a scintillation counter.

The requirement for residual ATP separation is the major drawback of this method. Additional assay considerations (both negative and positive):

- generates _____ waste
- non-continuous, meaning that it is necessary to take time points if you need kinetic data.
- General and sensitive. The assay works for _____ kinase.

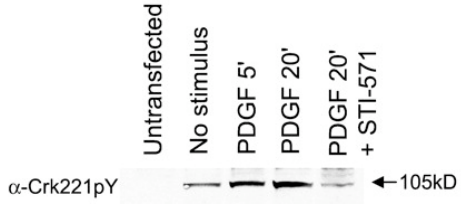
Note: ^{32}P incorporation (or other in-vitro) assays can be applied to cell lysate if the kinase of interest is first purified from the cell lysate by _____.

Immunoprecipitation is the use of an _____ to specifically bind a target protein and provide a handle for protein isolation.



B) Gel Analysis with a Phospho-specific Antibody: Western blot detection of a phosphorylated substrate

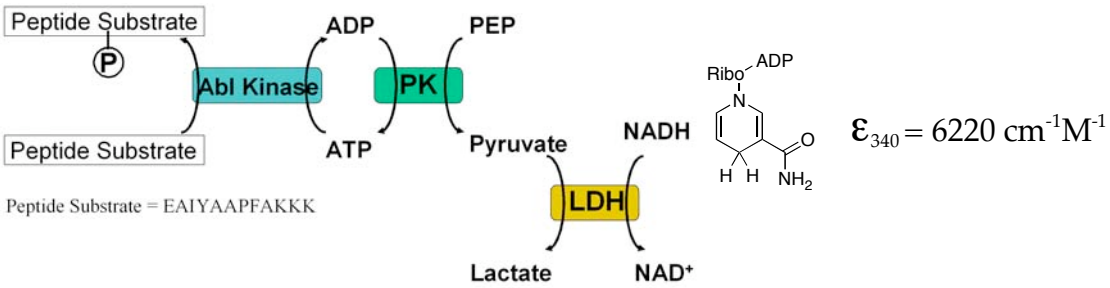
Ie. Detection of phosphorylated Crk (an Abl substrate) from cells with increasing concentrations of PDGF (Ting A. Y. et.al. *PNAS* **98**,15003-15008 (2001)).



Assay considerations (depending on your specific application)

- Non-continuous.
- Often used qualitatively
- Highly _____ and often used with crude cell lysate

C) Coupled Phosphorylation Assay (Session 13 and 14): (Indirect) detection of ADP formation/ATP consumption



$\varepsilon_{340} = 6220\ cm^{-1}M^{-1}$

Through two enzymatic reactions, Abl kinase activity is coupled to the conversion of _____ to _____.

The specific activity of the kinase can be calculated based on the decrease in NADH absorbance at _____ nm over time. The ε_{340} of NADH = 6220 $cm^{-1}M^{-1}$.

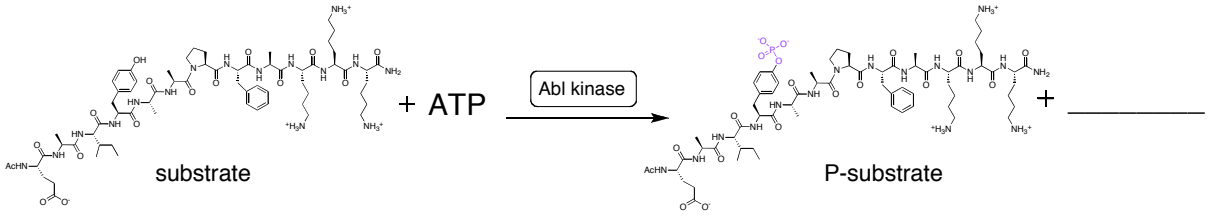
Extinction coefficient (ε): A molecule's extinction coefficient is a measure of the extent to which the molecule _____ light at a given wavelength. ε is typically reported for the wavelength of maximum absorbance (ε_{max}) in units of $cm^{-1}M^{-1}$.

Beer's Law: Abs = εcl	where c = concentration, and l = pathlength of the cuvette

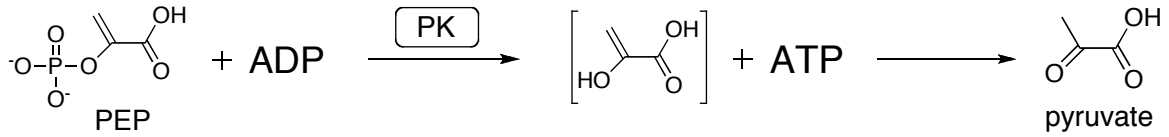
Beer's law allows you to use ε to calculate the _____ of a sample in solution by measuring the Abs at a given wavelength.

Details of the coupled kinase phosphorylation assay

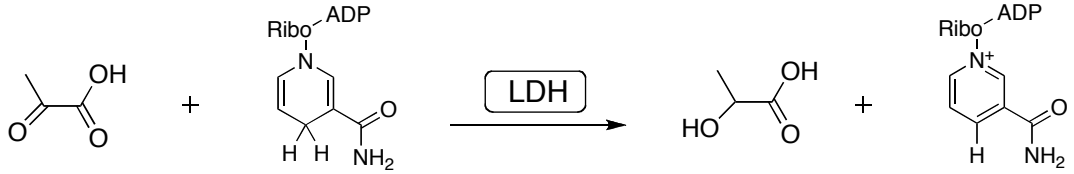
The protein kinase catalyzes the transfer of a phosphate group from ATP to a T, S, or Y on the peptide substrate. For our Abl assays, the substrate is EAI___AAPFAKKK.



Pyruvate kinase (_____) transfers a phosphate group from phosphoenolpyruvate (PEP) to ADP to form _____ and ATP.



Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate to _____ with concomitant oxidation of the coenzyme _____ to NAD^+.



You will monitor the _____ of NADH absorption at _____ nm to quantify the _____ of wt and H396P Abl kinase domain

1. in the absence of any inhibitors
2. with the Abl inhibitor Gleevec
3. with the Abl inhibitor Dasatinib

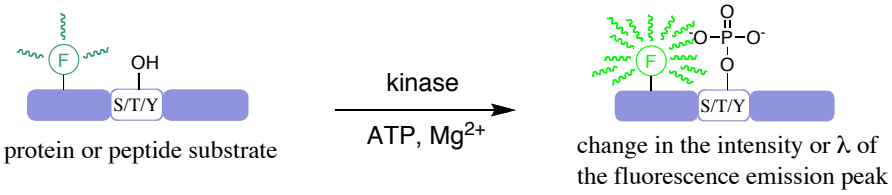
Specific activity in biochemistry is defined as the amount of product formed by an enzyme in a given amount of time. Specific activity is often reported as:

_____ (U) per ____ of enzyme,

(where 1 unit = 1 μmol of product formed per minute).

II. FLUORESCENT PEPTIDE-BASED PROBES

Detection of phosphorylated substrate



protein or peptide substrate

change in the intensity or λ of the fluorescence emission peak

F = environment-sensitive or chelation-enhanced fluorophore

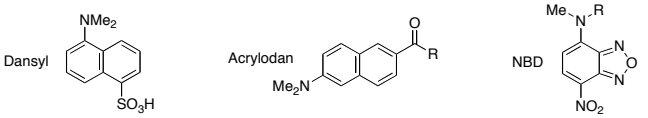
These probes comprise a substrate peptide that includes an amino acid recognition sequence for a specific kinase and an appropriately positioned _____-sensitive or _____-enhanced fluorophore.

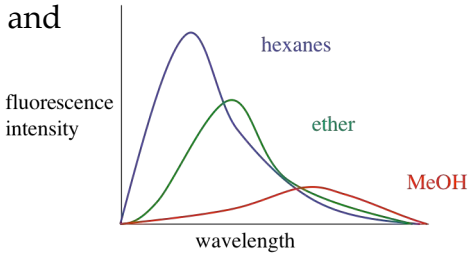
Upon phosphorylation of the substrate probe, the flurophore's emission properties change, enabling detection.

A) Environment-Sensitive Fluorophores

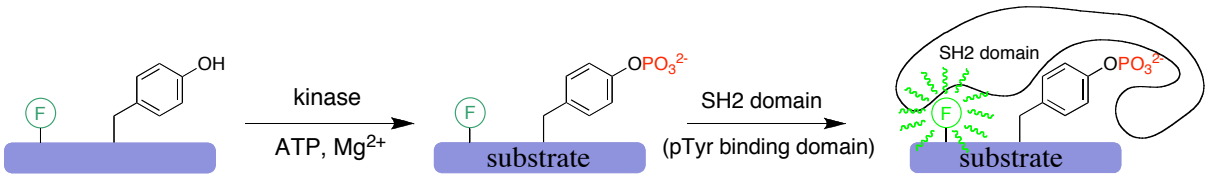
The signal intensity and maximum emission wavelength of environment-sensitive fluorophores (also referred to as solvatochromic dyes) are affected by the _____ of the fluorophore's immediate environment.

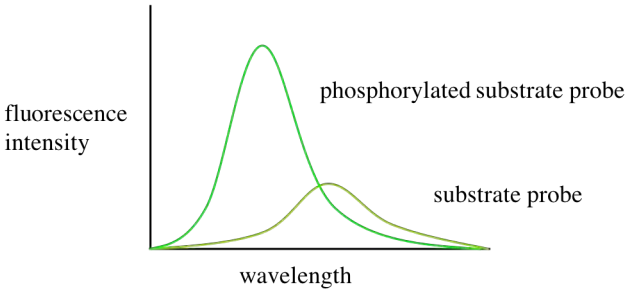
For example, the fluorophores below are quenched and red-shifted in polar environments.





A phosphorylation-induced conformational change or intramolecular _____ event that results in a change in the fluorophore's local environment can be detected by a change in fluorescence intensity.

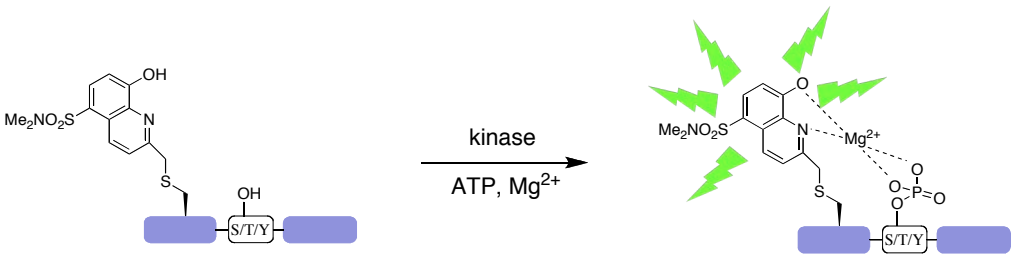




B) Chelation-Enhanced Fluorophores

Chelation-Enhanced Fluorophores demonstrate dramatic fluorescence intensity increases upon metal chelation.

In CHEF-based kinase activity probes, the probes have low affinity for Mg^{2+} in the non-phosphorylated form. Once phosphorylated the binding affinity increases drastically, resulting in a ____ to _____ fold fluorescence enhancement.



Protein or peptide substrate with a chelation-sensative fluorophore (ie. "Sox")

This is an ideal assay for cell lysate applications. Adjustments in the sensors to optimize detection with lower levels of $MgCl_2$ (initial conditions required 10 mM $MgCl_2$, above physiological levels) will enable in-vivo applications.

Advantages to Peptide-Based Probes

- Assays enable continuous readings (no work-up steps prior to detection) making them ideal for kinetic studies.
- Peptide probes are amenable to large-scale _____.
- Compared to proteins, peptides are incredibly _____ for handling/storage.
- Analogs to sense different kinases and explore different sensing techniques are readily accessible.
- Fluorescence change upon phosphorylation in many peptide-based sensors greatly exceeds 100%.

Disadvange to Peptide-Based Probes for In-vivo Applications

- It is challenging to uniformly introduce peptide probes into cells for in-vivo studies. (Most peptides are not cell-permeable.)

Methods to introduce fluorescent probes into cells

- _____
- Protein transduction domains (typicallyArg-rich peptide sequences) that are fused to target peptides or small proteins and render them cell permeable.
- Introduction of a non-natural amino acid during protein expression (hijacking RNA translational machinery) for a genetically-encoded probe.

III. IN-VIVO PROTEIN-BASED PROBES (ie. FRET)

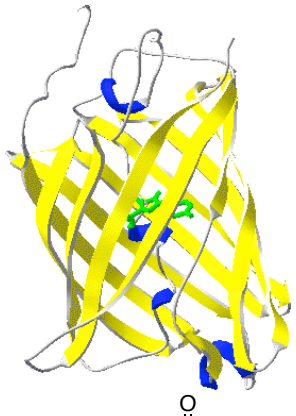
Protein probes for sensing kinase activity typically employ fluorescence resonance energy transfer (_____) to sense a conformational change induced by protein phosphorylation.

FRET is a distance-dependent interaction between a donor and an acceptor fluorophore and is used as a "molecular ruler".

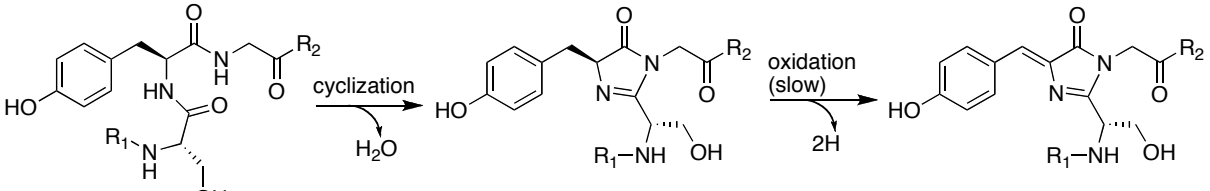
When the donor and acceptor fluorophores are in close proximity (10-100 A), the acceptor fluorophores is excited by the donor emission and emits a signal*.

*In FRET, the emission spectrum of the donor must overlap with the absorption spectrum of the acceptor.

Green Fluorescent Protein (GFP) and related fluorescent proteins



- _____-amino acid GFP first isolated from the Jellyfish *Aequorea Victoria*
- The chromophores of GFP and related fluorescent proteins arise from natural amino acids, so they can be expressed _____.



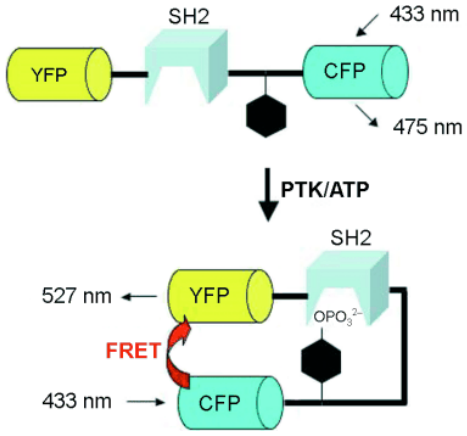
_____65 - _____66 - _____67

For (S65T) GFP: excitation: 488 nm; emission: 507 nm
The denatured GFP protein is NOT fluorescent. GFP is _____-sensitive.

Protein engineering has led to the development of a rainbow of other GFP analogs with a range of excitation and emission wavelengths. All are _____ , but also _____ (~230 amino acids).

FRET-based phosphorylation sensors: Detection of a phosphorylation-induced _____ change

For example, below is a cartoon of a FRET-based sensor for protein tyrosine kinases (PTK) using the CFP/YFP FRET pair.



The major advantage to FRET-based probes is that they can be expressed in cells.

However, the large _____ of FRET fluorophore pairs is a concern, particularly in comparison to small-molecule fluorophores.

Also, most FRET-based probes demonstrate a fluorescence increase of less than _____% following phosphorylation.

IV. LABORATORY REPORT GUIDELINES

Grading Rubric for the Laboratory Report / Lab Work: **50 points total**

Laboratory Report: **42 points**
- Abstract: **5 points**
- Introduction: **10 points**
  - Explanation of purpose/relevance: (5)
  - Demonstration of understanding: (5)
- Results: **5 points**
  - Organization/Clarity: (5)
- Discussion: **10 points**
  - Interpretation of data: (5)
  - Demonstration of understanding: (5)
- Appendices: **12 points**
  - PyMol Structure Worksheet: (4)
  - Materials and Methods: (4)
  - Supplementary Info: (4)

Laboratory Work: **8 points**
- Overall technique: (4)
- Effort and attitude: (4)

Remember: You want to impress us with your understanding of the background, the techniques, and the chemistry involved.

General Outline of the Module 4 and 5 Laboratory Report:
Please use the following subheadings in your report.

Abstract
This should provide a concise summary of the purpose of the report, major experimental data obtained, and major conclusions. The length should not exceed 250 words.

Introduction
This serves to introduce the reader to the report that follows. The introduction should also emphasize the relevance and importance of the experiment.
Your introduction should include:
- Any background information that is required to orient the reader to the experiment.
- The purpose of the experiment (why is it important, why is the information helpful/needed)
- An overall statement of the scope of the experiment and any of its limitations

Do **not** use the first person. The introduction should include references from any pertinent literature if necessary (the first journal reading assignments from Lectrue #1 should provide sufficient information for your introduction). Please reference as in *Nature*.

Experimental Results

Describe the results. Refer the reader to any specific figures, tables, or appendices. All essential figures **must** go in this section. The goal of this section is to simply report the results. DO NOT discuss or interpret the data here.

Discussion (of Results)
In this discussion you must interpret the results reported in the section above. State how these results compare with anticipated results or theory. Also, provide suggestions or explanations for any deviations. You may also discuss the sources of any error in this section.

Conclusions
This is a BRIEF summary of the project goals and major findings. This should be **one** short paragraph, concise and direct.

References
Again, please follow *Nature* reference formatting. It is ok to have only 1 reference.

Appendices (MUST have the following three):

1) PyMol structure viewing worksheet (completed in laboratory Session 15)

2) Materials and Methods

Include a brief summary of the methods used to obtain the results discussed in the main body of the paper. You should not recopy the entire lab manual protocol. Brevity and clarity are **key**.
For example one might write:

Isolation of wt AblK plasmid
The wild-type AblK plasmid was isolated from a 12-mL overnight culture of DH5-α cells transformed with the wild-type AblK plasmid. A Qiagen Miniprep kit was used to extract the plasmid from the cells. Final DNA concentration was determined by measuring the absorbance at 260 nm.

Protein concentration determination
A Bio-Rad BCA assay was used to determine the concentration of the purified, dialyzed AblK protein. The standard curve was obtained using BSA dilutions (0.9, 0.7, 0.55, 0.4, 0.2 ug/uL) and measuring the absorbance at 595 nm.

3) Supplementary Data

This section is for any non-essential data/figures that aren't included in the lab report (such as std curve fits, absorbance readings, sequence information, etc.)

There is no length requirement or limit. However, please be as **concise and direct** as possible while still achieving all of the criteria outlined above. Remember that you want to impress us with your understanding of the background, techniques, and the chemistry involved.