Announcements

- Chapter 6&8 post-lab write-up due on week of Feb 25-Mar2 (next week).
- Chapter 8C gel image – you will receive a link to the files from your TF. There will be 2, one of your samples and one of the ladder. You will want to overlay these two images to conduct the analysis (you can do this in Photoshop or similar programs).
- Ch 6 gel images – you can convert these to TIFF/PNG on the computer attached to the imager – make sure **you can open your file on your computer before you leave lab this week.**

Chapter 9A: Protein Modification

Objectives

- To learn about the enzyme ketohexokinase (KHK) and its role in fructose metabolism
- To modify sulfhydryl group (thiol group) of cysteines residues in a protein using DTNB (Ellman's Reagent)
- To measure KHK activity using a "coupled assay"
- To learn how modification of specific cysteine residues affects KHK activity

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Procedures

- To use **DTNB** to ***modify cysteines groups in KHK***
- To use a **coupled assay for KHK activity** to ***investigate effect of DTNB on enzyme activity***
- To **vary concentration of coupling enzymes (Pyruvate Kinase)** to ***investigate KHK coupled assay***

Various methods to study enzymes

- Testing optimal buffer conditions
  - **Chapter 3 & 4 - LDH in Tris vs KPO_4 buffer**
- Steady-state kinetics with various inhibitors; measure K_m, k_{cat}, K_i
  - **Chapter 4**
- Point mutation studies (*later in Biochem II*)
  - **Chapter 11C**

- **<u>Protein modifications</u> (Chapter 9)**

Protein Modifications

- **USE: Probe role of particular amino acids in protein structure and function**
- Commonly used reagents are specific for one amino acid or functional group

Reagent	Residue	Detection
2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent)	Tryptophan	410 nm
N-bromosuccinimide	Tryptophan	260/280 nm
Phenylisothiocyante (Edman's Reagent)	Amino-terminal	Release of a PTH-amino
Iodoacetic acid	Cysteine	Carboxymethyl derivati
*N-ethylmaleimide (NEM)	Cysteine	Derivatives of NEM
*5,5'-Dithiobis(2-nitrobenzoic acid) (Ellman's Reagent - DTNB)	Cysteine	412 nm
Diethylpyrocarbonate (DEPC)	Histidine	240 nm
Imidates	Lysine	Derivatives of imidates
2,4,6-trinitrobenzenesulfonic acid (TNBS)	Lysine	420 nm

*Reagents used in this experiment; although the NEM will be attached to fluorescein such that can be detected by fluorescence.

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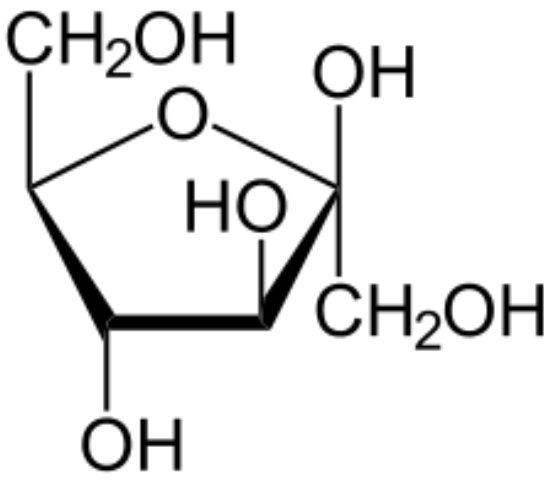
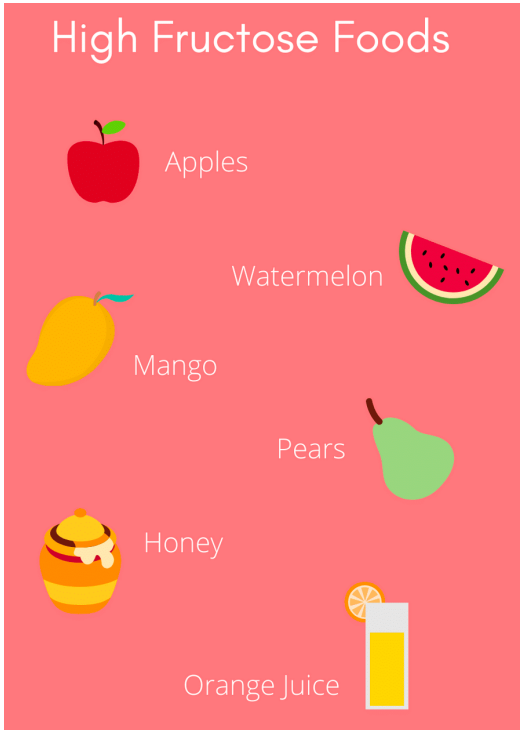
- **ADVANTAGES:**
  - Cause an easily observed chemical change in the protein
  - Can monitor amount of incorporation into protein and change in enzyme activity
- **DISADVANTAGES:**
  - Can only observe accessible residues
  - Can cause side reactions with other residues

Fructose

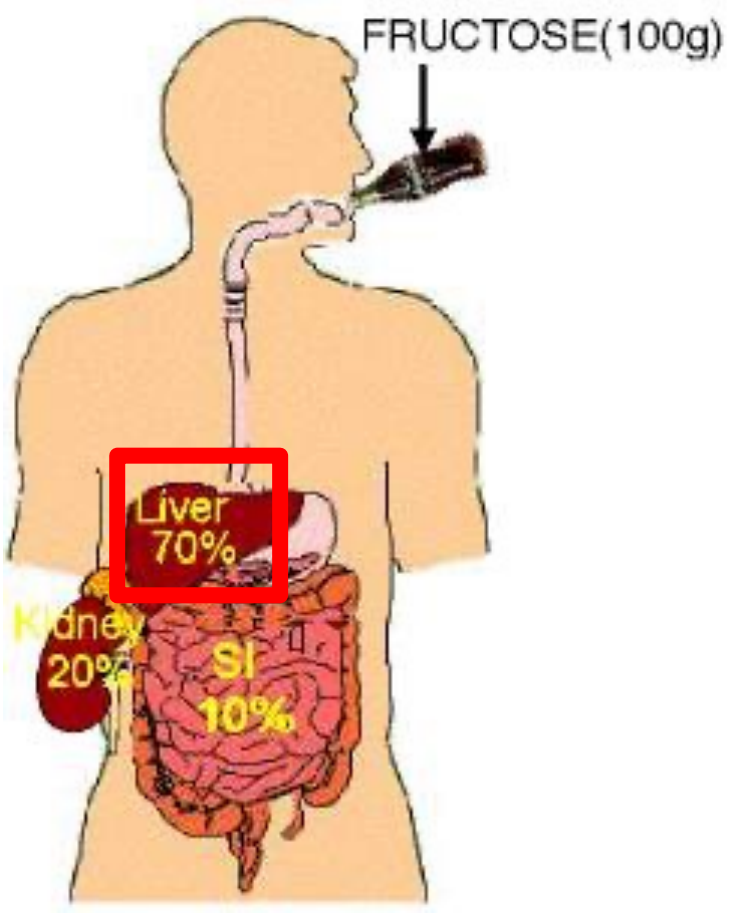




- Monosaccharide
- Food additive in many processed food

https://fodmapfriendly.com/blogpost/how-to-enjoy-fruit-with-fructose-intolerance/

Fructose Metabolism



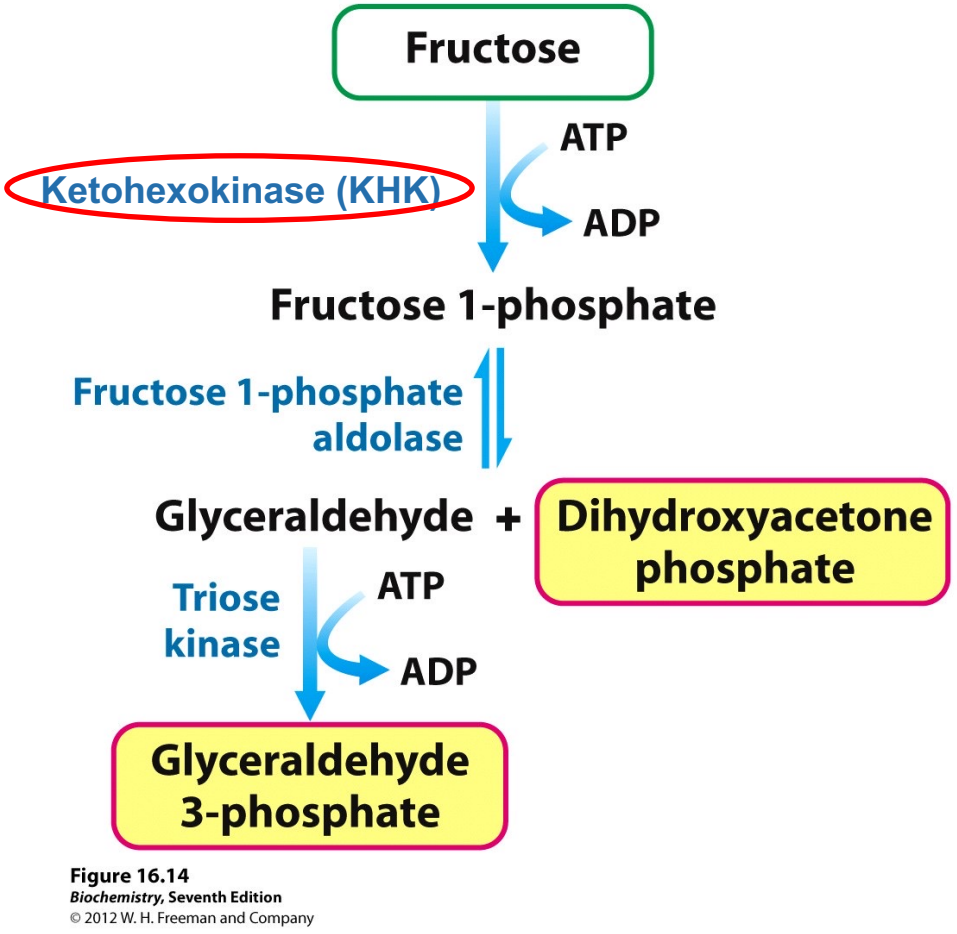


Figure 16.14
Biochemistry, Seventh Edition

http://www.bu.edu/aldolase/HFI/hfiinfo/wt.html

Inborn Errors in Fructose Metabolism

- **Essential Fructosuria (EF)** – deficiency in KHK
  - Clinically benign condition
  - 1:130,000 have EF but numbers likely higher since people affected are asymptomatic

- **Hereditary Fructose Intolerance (HFI)** – deficiency in Aldolase B
  - Potentially lethal
  - Ingestion of fructose causes an accumulation of fructose-1-phosphate which, over time, results in the death of liver cells

Ketohexokinase (KHK)

- KHK exists in two isoforms – KHK-A and KHK-C

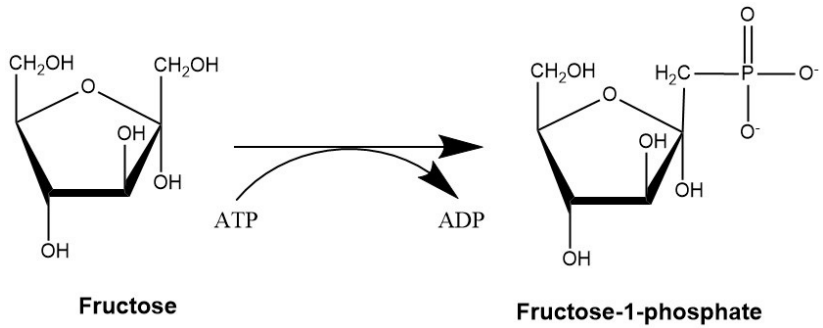
KHK-A

- Ubiquitous expression
- K_M = 7mM (Fructose)
- Role not clearly defined

KHK-C

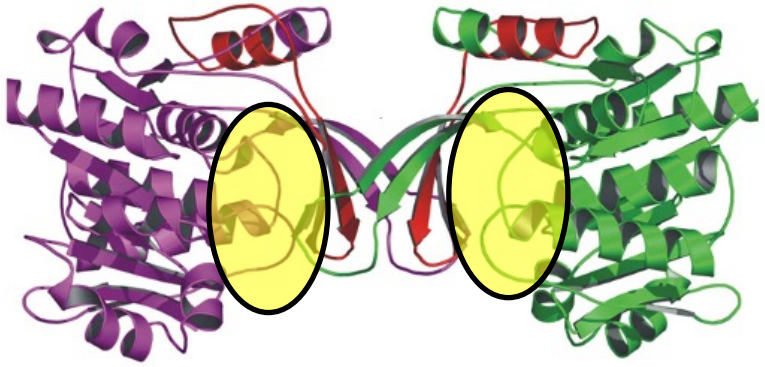
- Liver, Kidneys, Intestines
- K_M = 0.8mM (Fructose)
- Physiological role in fructose metabolism

- Exist as a dimer
- **There are many Cys residues, the role of which are unknown.**



Fructose

Fructose-1-phosphate

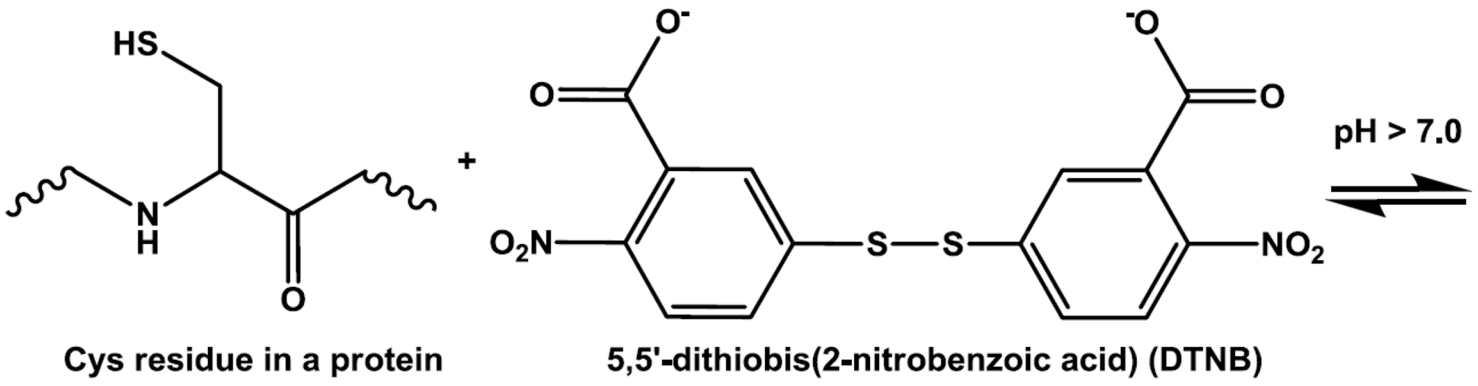


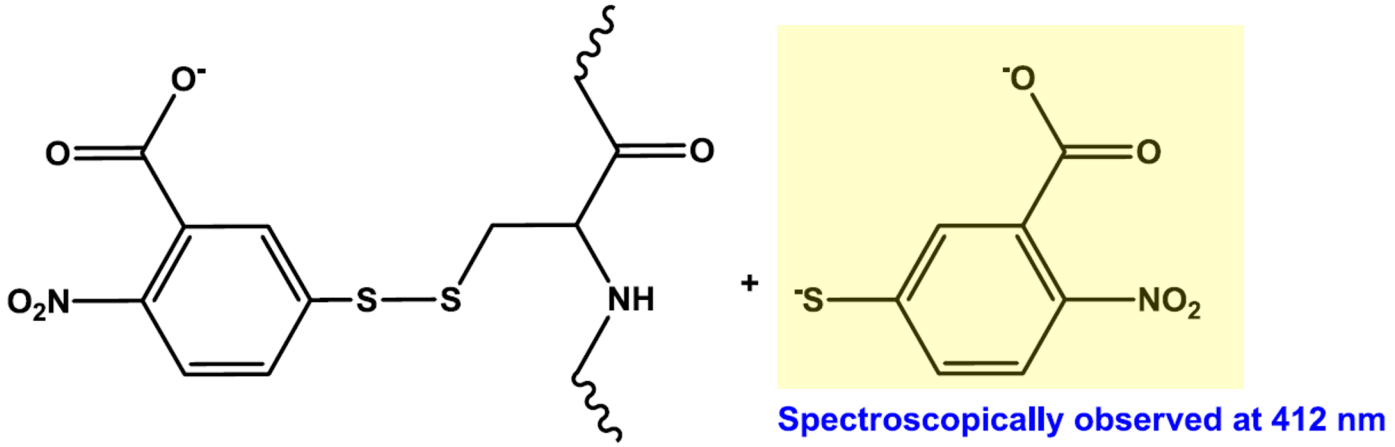
Human KHK Structure

Trinh et. al. (2009)

5,5’-Dithiobis(2-nitrobenzoic acid)

- DTNB reacts with Cys thiol group





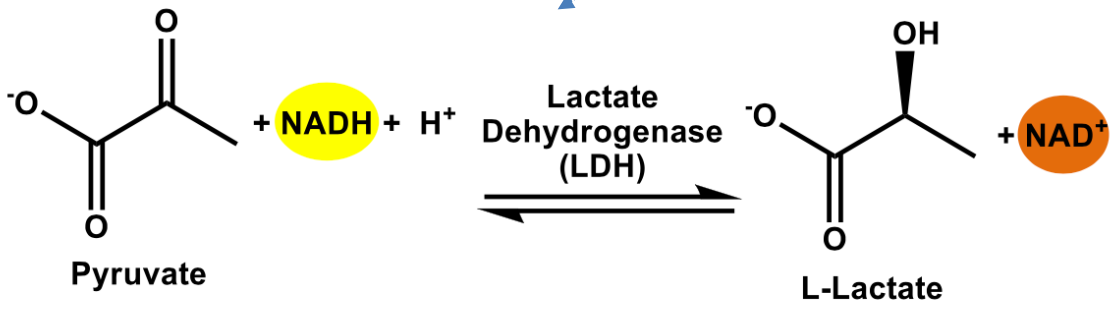
- Can quantitatively determine incorporation of DTNB by $A_{412\ nm}$

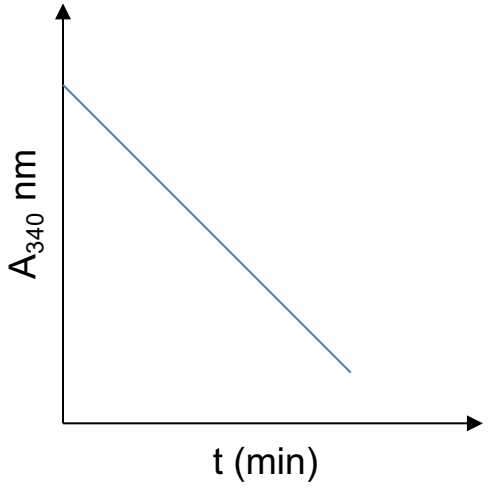
5,5’-Dithiobis(2-nitrobenzoic acid)

- Can observe effect of DTNB on activity
- Can denature enzyme and compare native and unfolded states
  - Are the number of Cys residues the same?
  - If not, why?
    - **Buried residues may be inaccessible in native state**

Measuring KHK activity

Recall Chapter 3…

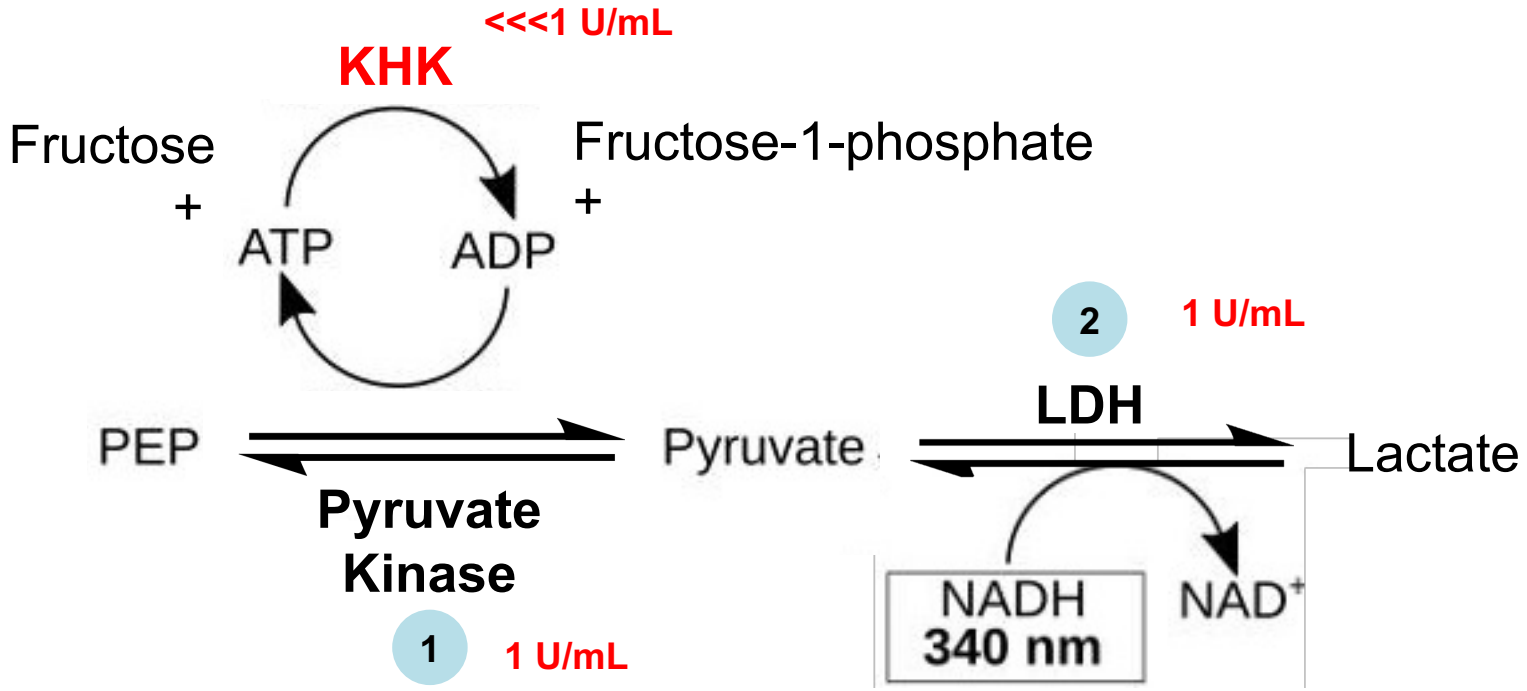




NADH has a visible absorbance at **340 nm** – can follow its rate of oxidation as reaction progresses → ΔA_{340}/min

The Coupled Reaction for KHK activity

- Coupling multiple enzymes in sequential reactions
- PK and LDH must be in excess of KHK so that KHK activity is rate limiting *Why?*



Measure KHK activity rather than PK or LDH activity!
Initial rate of reaction will be proportional to [KHK]

General overview of Chapter 9A

Part I: DTNB Modification Reactions

- Modify exposed Cys residues on KHK with DTNB
- Use denaturants (SDS) to test for buried Cys
- Use substrates to test for protection of potentially other important Cys
  - *Is there a difference in DTNB readings among these reagents? Why?*

Part II: KHK Activity Assay

- Test the activity of four samples of DTNB modified KHK
  - *Is there a difference in KHK activity compared to controls? Why?*

Part III: Coupled Assay

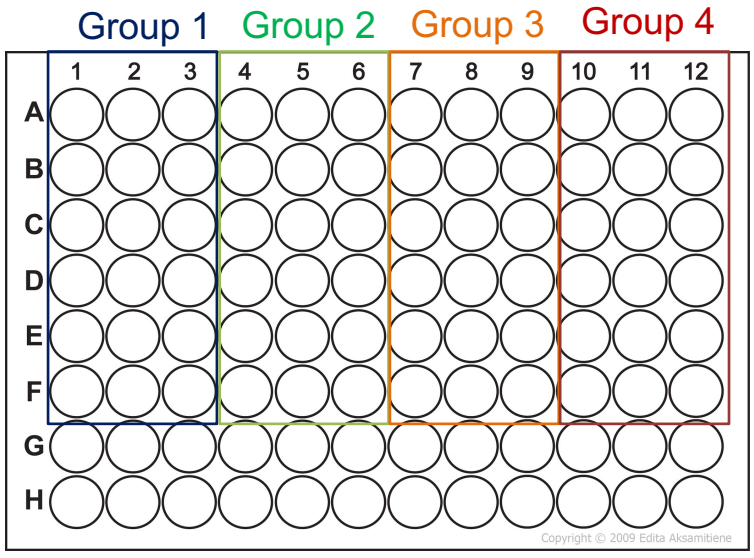
- Test different amounts of **PYRUVATE KINASE** to check if the coupling enzyme of the coupled assay are in excess.
  - *Why is this important?*

Chapter 9A: Procedure

Part I: DTNB Modification Reactions

- 6 reactions to set up per group (triplicates) in a 96-well clear bottom plate

A: Negative control: DTNB only
B: Positive control: DTNB + CysNAc
C: KHK only (positive control for KHK activity)
D: KHK + DTNB
E: KHK + DTNB + ATP
F: KHK + DTNB + SDS (also negative control for KHK activity)



We only have 2 plate readers so 4 groups need to share a 96-well plate

Chapter 9A: Procedure

- Prepare the 6 reactions according to the table below in a 1.5 mL microcentrifuge tube

- Prepare volumes needed for ~3.5 reactions (slightly >3) to account for pipetting errors

Fold excess over reaction's final conc.

TF will add!

Volumes used in each well***	BUFFER	KHK	CysNAc	ATP	SDS	H_2O	Volume	DTNB	Final Volume
Fold dilution from stock	2.5x	25x	10x	10x	10x	–	–	5x	
[Stock]	*	250 μM**	50 μM	10 mM	1 %	–	–	500 μM	
[Final] in Assay	*	10 μM	5 μM	1 mM	0.1%	–	–	100 μM	
A	100	–	–	–	–	100	200	50	250
B	100	–	25	–	–	75	200	50	250
C	100	10	–	–	–	140	250	–	250
D	100	10	–	–	–	90	200	50	250
E	100	10	–	25	–	65	200	50	250
F	100	10	–	–	25	65	200	50	250
Order of addition:	2	6	3	4	5	1			

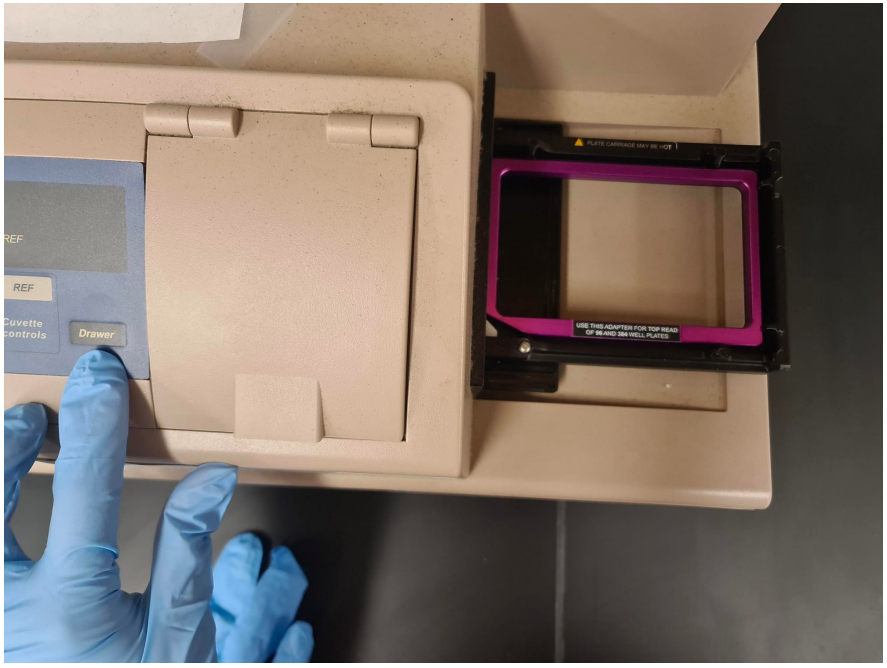
*Buffer is 62.5 mM Tris, pH 7.5, and 1.25 mM EDTA. This is the 2.5x stock which has concentrations in the 1x assay of 25 mM and 0.5 mM, respectfully. **the stock concentration of 250 µM is approximately 8 mg/mL. *** volumes in the table are all in µL

Do not add DTNB into reaction!

Chapter 9A: Procedure

- Record the columns that your TF assigned you to load your samples on the 96 well plate. For example, Row A-F Column 1-3
- Pipet the appropriate volumes into each well. Each well should contain 200 µL except for row C, which will not have DTNB. Row C samples should have 250 µL instead
- Inform your TF once you have finished loading so that the next group can continue loading
- Once 4 groups have finished loading, your TF will bring your 96-well plate to the Molecular Devices plate reader located either in SCI-358? or SCI-427 You are welcome to follow your TFs to see how the plate readers are operated

Chapter 9A: Procedure



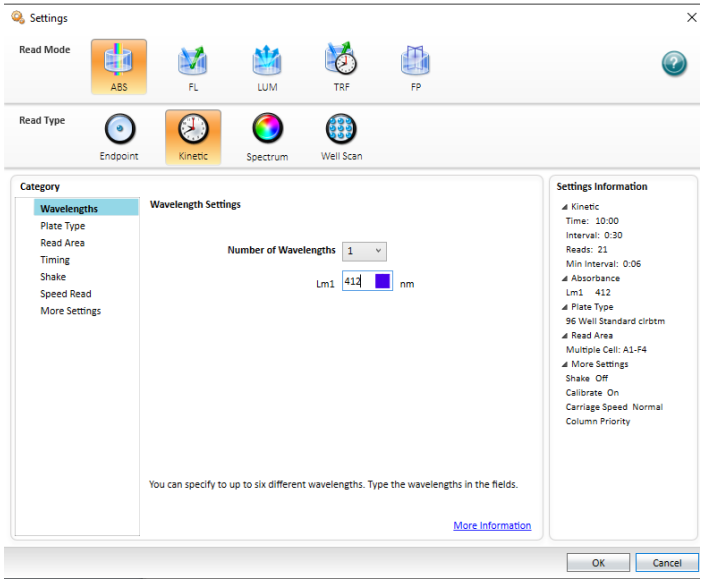
Press on the “drawer” button on the plate reader to open the sample tray.

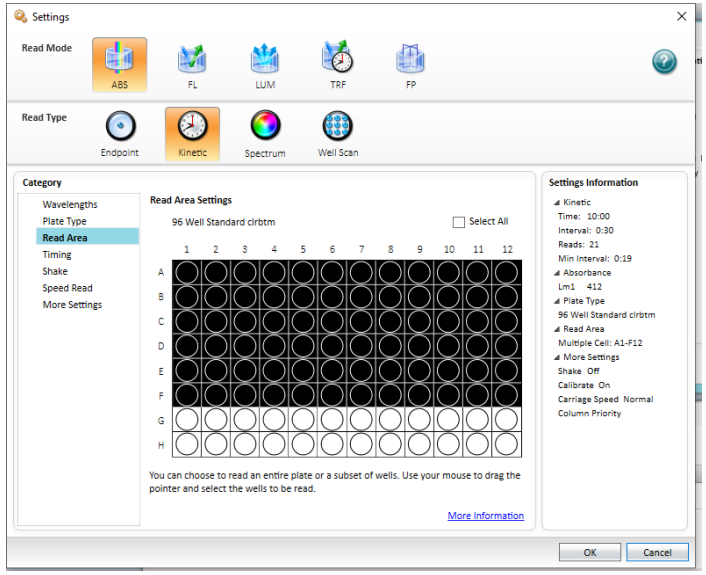
Remove the plate lid and load the plate into the drawer. **Please ensure that the plate is properly aligned in the drawer before you close it!**

Chapter 9A: Procedure

- Open the **SoftMax Pro** software on the computer

- The plate readers will be set in the **ABS (absorbance) read mode** and **Kinetic read type** for a total run time of 1 h, 30s interval, 412 nm for data collection





- Your TF will use a multi-channel pipet to add 50 µL 0.5 mM DTNB to samples. Check that there are no bubbles before clicking on the **READ** button

- Once the run has completed, remember to export the file by columns and in .xls format. **Save it on a flash drive.**

Chapter 9A: Procedure

- Before measuring activity of your samples in Part II, you need to quench your DTNB reaction samples
  - Add 100 µL of **any one** of the triplicate KHK-containing reactions in row C, D, E and F into tubes containing 100 µL 100 mM β-mercaptoethanol. **Put tubes on ice!**

- Cover the entire 96-well plate with the plate lid, parafilm. We will keep the entire plate at 4 °C until Chapter 9C where we can measure protein concentration

- If you have extra quenched aliquots samples, please save it as a backup for Chapter 9C

Chapter 9A: Procedure

Part II: KHK Activity Assay

- Make 2x KHK cocktail for **at least 16 assays**:

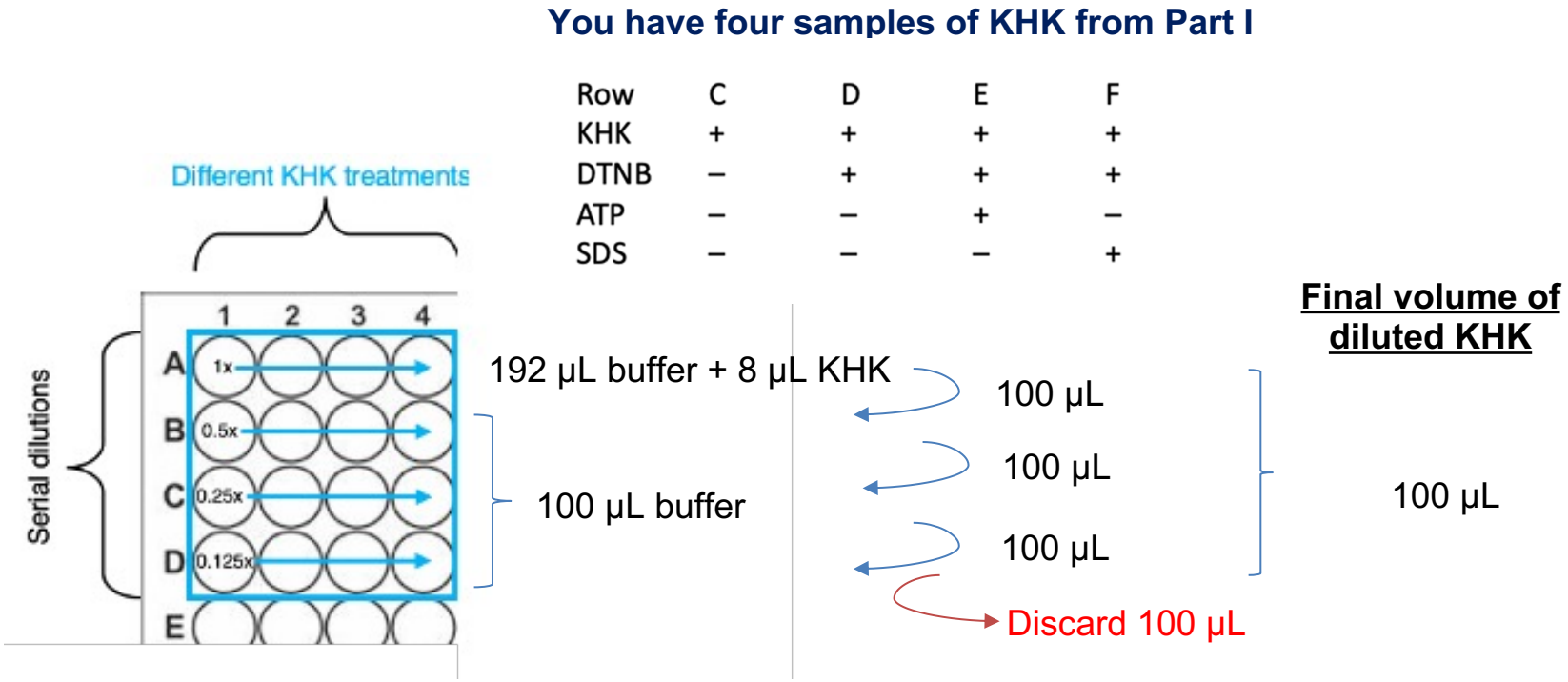
	Reagent	Volume/2x assay cocktail (µL)	Final Concentration
1	TEA-HCl, pH 7.4, 1 M (30x)	133	33 mM
2	KCl, 4 M (40x)	100	100 mM
3	NADH, 30 mM (100x)	40	300 µM
4	$MgCl_2$, 1.2 M (200x)	20	6 mM
5	Phosphoenolpyruvate (PEP), 0.133 M (100x)	20	1.33 mM
6	ATP (K_m = 0.13 mM), 65 mM (50x)	80	1.3 mM
7	Fructose (K_m = 0.25 mM), 500 mM (50x)	80	10 mM
8	Pyruvate kinase, 1000 U/mL (1000x)	4	1 U/mL
9	Lactate dehydrogenase, 1000 U/mL (1000x)	4	1 U/mL
10	H_2O	1519	
	Total volume of KHK 2xcocktail	2000	

Combine these components as part of a "cocktail." Keep cold until ~5 min prior to assay.

- The reaction is prepared at 2x as you will eventually add 100 µL appropriately diluted KHK to 100 µL 2x cocktail to give 1x
- You can prepare the 2x cocktail while waiting for your DTNB assay, but keep it on ice
- ~5 minutes before you start measuring activity, leave it at room temperature

Chapter 9A: Procedure

- Take a **new** 96-well clear bottom plate (per group)



- Prepare dilutions of each of your four quenched KHK samples
  - ➢ Pipet 192 µL 10 mM HEPES pH 7.4, 0.5 mM DTT **(EDB)** into Row A, columns 1-4 (50-fold dilution) and 100 µL into Rows B to D, columns 1-4
  - ➢ Add 8 µL of quenched KHK into A1 to A4. Mix well
  - ➢ Pipet 100 µL from row A and aliquot into row B. Mix well
  - ➢ Do the same from B to C, and C to D.
  - ➢ Discard 100 µL from row D

Chapter 9A: Procedure

- Bring your 96-well plate along with your 2x KHK cocktail and multi-channel pipette and tips to the plate reader
- The plate readers will be set in the **ABS (absorbance) read mode** and **Kinetic read type** for a total run time of 5-10 min, 10 s interval, 340 nm for data collection
- Once you are ready to begin, aliquot 100 µL of 2x cocktail into each well and click on the **READ button**
- Again, remember to save and export your file in .xls format

Chapter 9A: Procedure

Part III: Coupled Assay

- Varying [Pyruvate Kinase] now instead of [KHK]
- Make 2x PK cocktail for **at least 12 assays**:

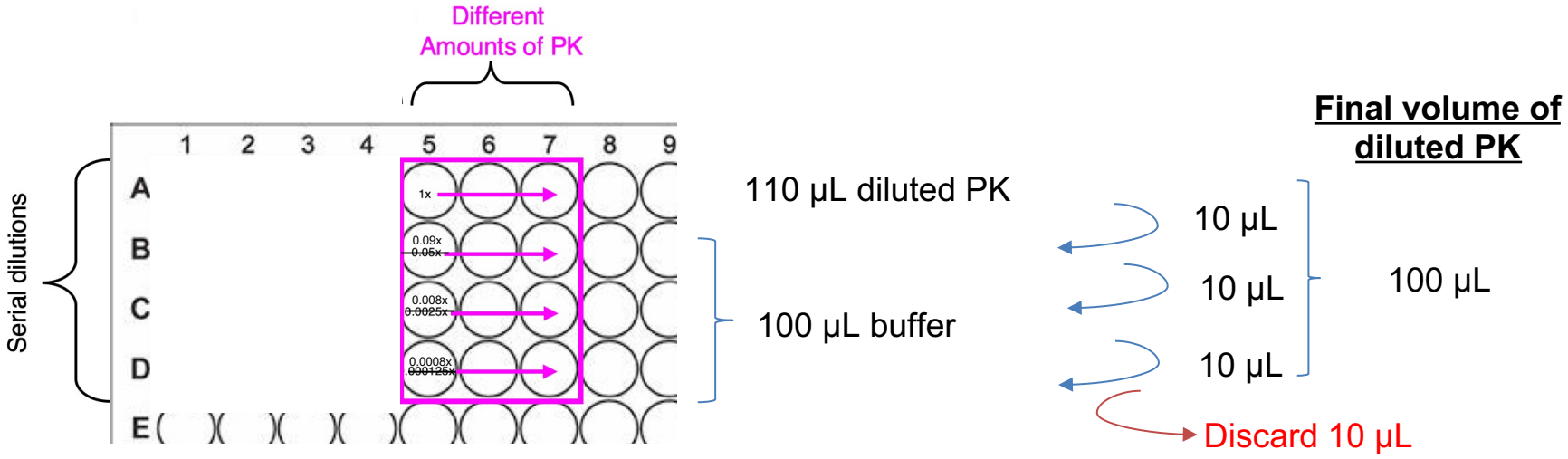
	Reagent	Volume (µL)/assay (µL)	Final Concentration
1	**TEA-HCl, pH 7.4, 1 M (30x)**	100	33 mM
2	**KCl, 4 M (40x)**	75	100 mM
3	**NADH, 30 mM (100x)**	30	300 µM
4	**$MgCl_2$, 1.2 M (200x)**	15	6 mM
5	**Phosphoenolpyruvate (PEP), 0.133 M (100x)**	15	1.33 mM
6	**ATP (K_m = 0.13 mM), 65 mM (50x)**	60	1.3 mM
7	**Fructose (K_m = 0.25 mM), 500 mM (50x)**	60	10 mM
8	**Lactate dehydrogenase, 1000 U/mL (1000x)**	3	1 U/mL
9	**Ketohexokinase (KHK),** 5 µM (50x)(from rxn C)	25	0.0027 U/mL
10	**H_2O**	1117	
	Total volume of PK 2xcocktail	1500	

Combine these components as part of a "cocktail." Keep cold until ~5 min prior to assay.

- You can prepare the 2x cocktail while waiting for your DTNB assay, but keep it on ice
- ~5 minutes before you start measuring activity, leave it at room temperature

Chapter 9A: Procedure

- Use the same plate for Part II, use rows A to D, column 5 to 7



- Prepare dilution of Pyruvate Kinase
  - ➢ Pipet 2 µL of stock PK and add to 1 mL EDB
  - ➢ Add 110 µL of diluted PK into Row A columns 5-7

- Pipet 100 µL of EDB into wells in Rows B to D, columns 5-7
- Remove 10 µL of samples from Row A and add to Row B. Mix well
- Do the same from B to C, and C to D.
  - ➢ Discard 10 µL from row D

Chapter 9A: Procedure

- Bring your 96-well plate along with your 2x PK cocktail and multi-channel pipette and tips to the plate reader
- The plate readers will be set in the **ABS (absorbance) read mode** and **Kinetic read type** for a total run time of 10 min, 10s interval, 340 nm for data collection
- Once you are ready to begin, aliquot 100 µL of 2x PK cocktail into each well and click on the **READ button**
- Again, remember to save and export your file in .xls format

CH 9A Hazardous chemical waste

- Leftover buffers, enzymes, and all DTNB assays go into the mainstream waste (large carboy – might be in the fume hood)

CH 9A Lab Tips

- Work together in your preparation and setting up of the DTNB reactions in the 1.5 mL microcentrifuge tubes. Split up the work if you need to. (Important to always check the pipet tips volume match the pipet you are using!)

- Be efficient in the loading of wells on the 96-well plates since 4 groups are sharing a plate

- Check for bubbles. If necessary, use a needle to remove it.

- Prepare your reagent (BME) for quenching in advance in 1.5 mL microcentrifuge tubes. Add KHK samples to these pre-assembled tubes

- While waiting for DTNB reactions to finish, start preparing cocktail for activity assay and coupled assay – keep on ice until ~5 min before starting assay, leave at room temperature

- Remember to save your samples and your data!

Chapter 9 Week 1

Before the lab period, you should have:

Completed your Pre-lab Write-up and submit on Gradescope

Title, purpose and procedures

Remember to include:

Volumes of stock reagents in each DTNB reaction tube

Recipes for KHK activity assay (Part II) and Coupled assay (Part III)

Flow chart!!

At the end of lab, you should have:

Completed DTNB modification reactions (Part I)

Completed KHK activity assays (Part II)

Completed Coupled Assays (Part III)

Saved and put in fridge the 96-well plate from DTNB reactions and any remaining quenched aliquots for Week 3 – LABEL!

Record columns that your TF assigned you to load your samples on the 96 well plate for Part I

Saved all your data on a USB drive

Questions?

Discussion Quiz