Announcements

- Chapter 6/8 Laboratory Reports due on week of Mar 20-25.
- Chapter 8C gel image will be uploaded on Slack once we finish developing them.

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

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**

- **Protein modifications** (**Chapter 9**)

Protein Modifications

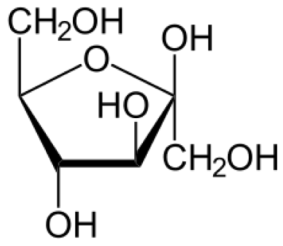
- **USE: Probe role of particular amino acids in protein structure and function**
- Commonly used reagents are specific for one amino acid or functional group

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

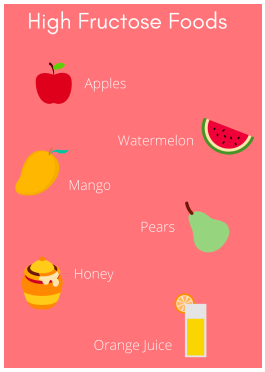
*Reagents used in this experiment; although the NEM will be attached to fluorescein such that can be detected by fluorescence.

- **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



β-D-Fructofuranose



- Monosaccharide
- Food additive in many processed food

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

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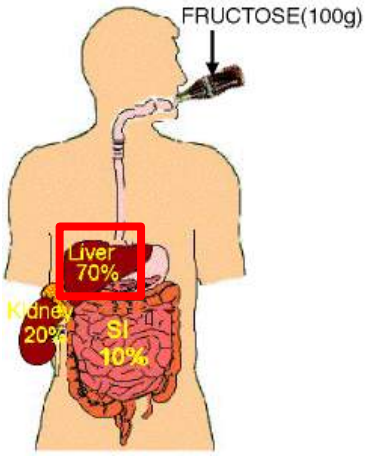
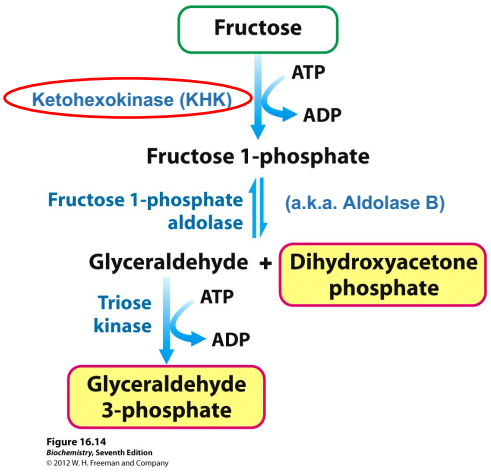
Fructose Metabolism





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

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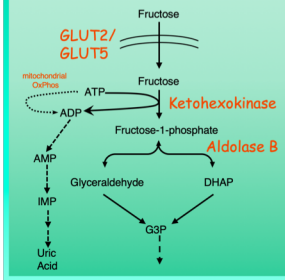
Inborn Errors in Fructose Metabolism

Clinical Correlations

Fructose Metabolism: Malabsorption, Essential Fructosuria & Hereditary Fructose Intolerance

- Three maladies arise due to fructose metabolism.
- Only the last one, deficiency of aldolase B, can lead to death. Even small amounts of fructose lead to severe hypoglycemia, jaundice, hemorrhage, hepatomegaly, uricemia, and kidney damage.
- Fructose can account for 30-60% of carbohydrate in the Western diet. Humans have a limited capacity to handle this sugar, and show the same ATP depletion after a sugar bolus. Use of fructose, sorbitol, and xylitol for parenteral nutrition (i.v.) has been discontinued to to liver damage.

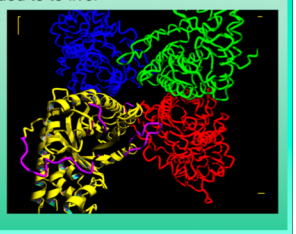
Giltzelmann *et al.*, 1995



Fructose Malabsorption 1 in 3

Hepatic Fructokinase Deficiency (aka Essential Fructosuria) 1 in 130,000

Hereditary Fructose Intolerance 1 in 20,000



Ketohexokinase (KHK)

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

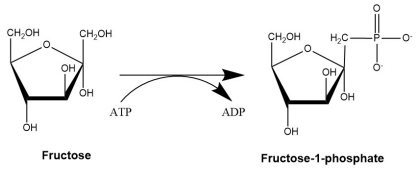
KHK-A

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

KHK-C

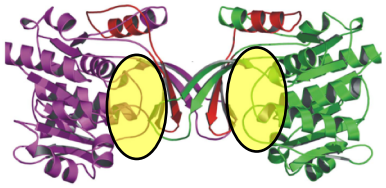
- Liver, Kidneys, Intestines
- K_M = 0.8 mM (Fructose)
- Physiological role in fructose metabolism

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



Fructose

Fructose-1-phosphate

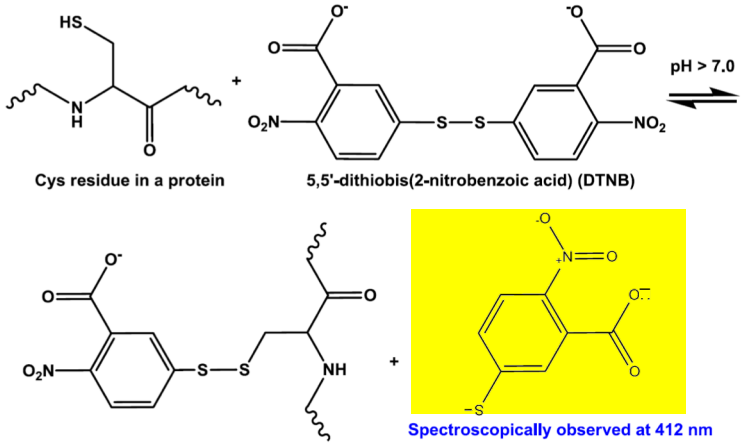


Human KHK Structure

Trinh et. al. (2009)

5,5'-Dithiobis(2-nitrobenzoic acid)

- DTNB reacts with Cys thiol group

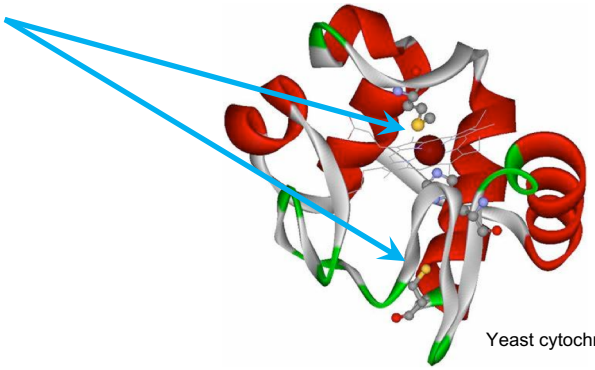


Spectroscopically observed at 412 nm

- 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**



Yeast cytochrome-C

Measuring KHK activity

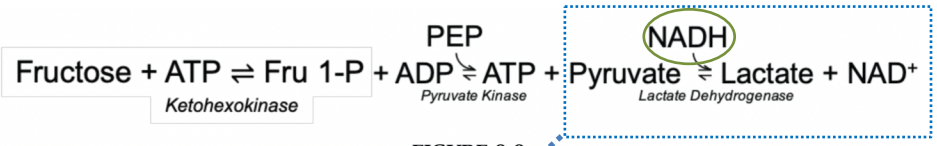
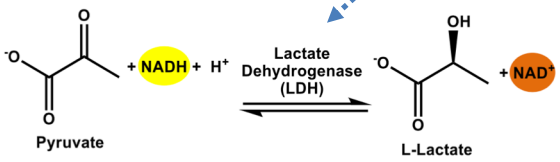
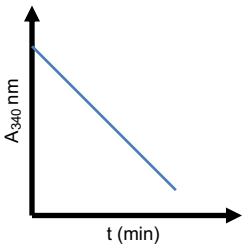


FIGURE 9-9

Recall Chapter 3…





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

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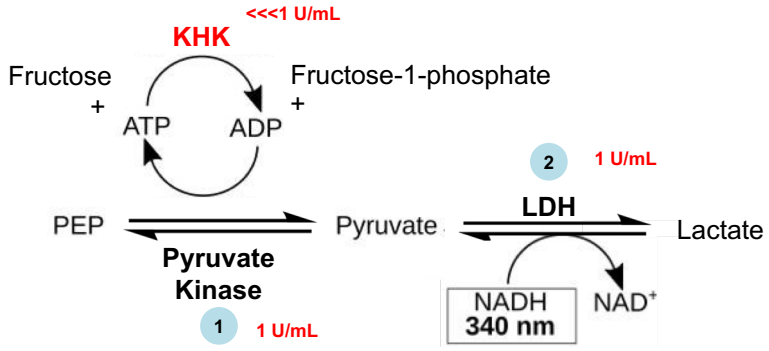
The Coupled Reaction for KHK activity

- 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]

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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?*

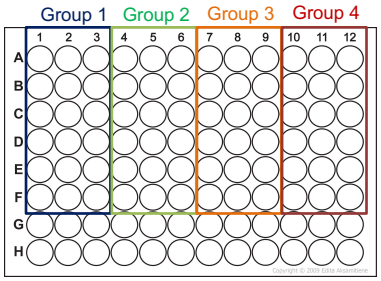
In-Class Activity:

Experimental Design

Chapter 9A: Procedure

Part I: DTNB Modification Reactions

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



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

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Chapter 9A: Procedure

- Prepare the 6 reactions according to the table below in a 1.5 mL microcentrifuge tube
- Prepare volumes needed for ~**3.2*** 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	10X	10x	10x	10x	–	These will change		
[Stock]	*	100 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	25	–	–	–	140	250	–	250
D	100	25	–	–	–	90	200	50	250
E	100	25	–	25	–	65	200	50	250
F	100	25	–	–	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!

*This is due to the limited amount of KHK we have

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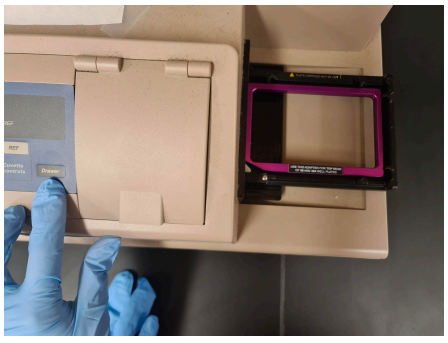
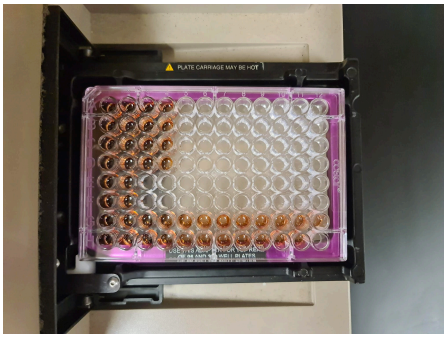
Chapter 9A: Procedure

- 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-156 or SCI-472 You are welcome to follow your TFs to see how the plate readers are operated

Chapter 9A: Procedure



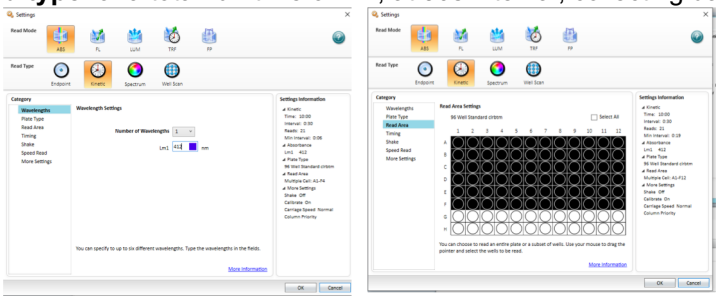
Press on the "drawer" button on the plate reader to open the sample tray.



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 if not already open.
- The plate readers will be set in the **ABS (absorbance) read mode** and **Kinetic read type** for a total run time of 1 h, at 30s interval, collecting data at 412 nm



- Your TF will use a multi-channel pipet to add 50 µL 0.5 mM DTNB to ALL samples for all 4 groups (except Row C). 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. You can either email the data to yourself or save it on a flash drive

Chapter 9A: Procedure

Part II: KHK Activity Assay (effect of DTNB on enzyme)

- 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!**
- Note, this is a 2-fold dilution of 100/250 of your KHK added to the Part I assay.
- Cover the entire 96-well plate with the plate lid, parafilm. We will keep the entire plate at 4 °C until we can measure protein concentration

Chapter 9A: Procedure

FIGURE 9-9

Part II: KHK Activity Assay

- Make a "2x KHK cocktail" for **at least 16 200 µL-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
Combined (8 µL)	8	Pyruvate kinase, 1000 U/mL (1000x)	4	1 U/mL
Combined (8 µL)	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 cocktail is prepared at 2x in all concentrations of reagents that are held constant. This will allow you to eventually add 100 µL of this 2X KHK cocktail to 100 µL of appropriately diluted KHK to give 1x concentrations of reagents.
- You can prepare the 2x cocktail while waiting for your DTNB assay, but keep it on ice.
- ~5 minutes before you start measuring activity, remove from ice and bring it to room temperature

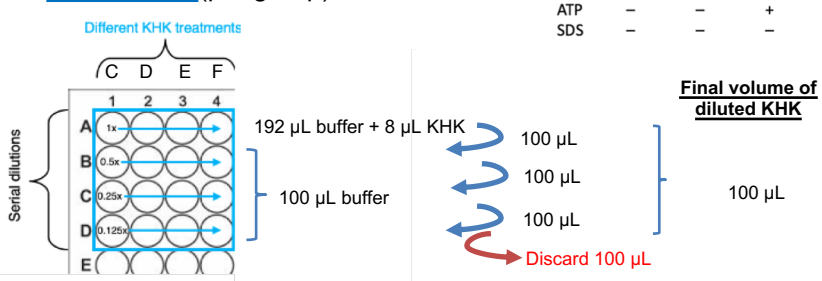
Chapter 9A: Procedure

Making serial dilutions of KHK

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

You have four samples of KHK from Part I

Row	C	D	E	F
KHK	+	+	+	+
DTNB	–	+	+	+
ATP	–	–	+	–
SDS	–	–	–	+



- 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 EDB into Rows B, C, and 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

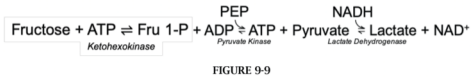


FIGURE 9-9

Part III: Coupled Assay

- Varying [Pyruvate Kinase] now instead of [KHK]
- Make 2x PK cocktail for **at least 12 200 µL-assays**:

	Reagent	Volume (µL)/assay	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; 1.5 U/mL)(from rxn C)	60	0.03 U/mL
10	H_2O	1082	
	Total volume of PK 2xcocktail	1500	

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

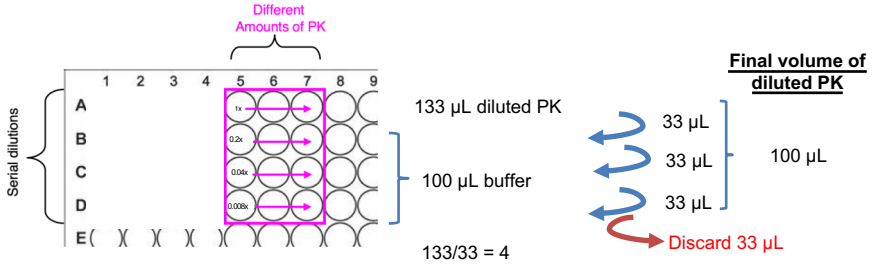
- You can prepare the PK 2x cocktail while waiting for your DTNB assay, but keep it on ice
- ~5 minutes before you start measuring activity, remove from ice and bring it to room temperature

Announce > Concepts > Procedure > Hazards > Tips > Clarification > End

Chapter 9A: Procedure

Making serial dilutions of PK

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



- Prepare dilution of Pyruvate Kinase
  - ➢ Pipet 4 µL of stock PK and add to 1 mL EDB
  - ➢ Add 133 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 33 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 33 µL from row D

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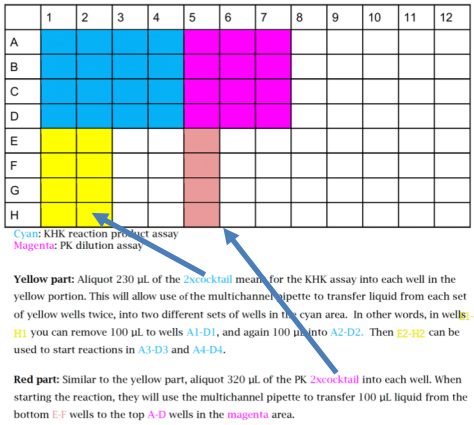
Chapter 9A: Procedure

Part II: KHK Activity Assay & Part III Effect of lower PK on Coupled Assay



- Put 2X cocktails in reservoir wells in Rows E–H (**230** & 320 µL in each well).
- Bring your 96-well plate 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, using 4 tips on the multi-channel pipet, aliquot 100 µL of 2x cocktail into each well (A-D) of each column, and click on the **READ button**
- Again, Once the run has completed, remember to export the file **by columns** and in .xls format. You can either email the data to yourself or save it on a flash drive

Announce Concepts Procedure Hazards Tips Clarification End

CH 9A Hazardous chemical waste

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

Announce Concepts Procedure Hazards Tips Clarification End

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 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!

Announce Concepts Procedure Hazards Tips Clarification End

Chapter 9A Clarification

- The KHK prep is less concentrated than suggested in the lab manual. It is 100 µM instead of 250 µM, so it is a 10X stock. You will need to re-calculate your recipes by adding more KHK and less water. Also, when making up your recipe, use 3.**2**-times, instead of 3.5 times what is required for 10 µM. To make is simpler and assure you have enough KHK for all reactions, just add 75 µL of your 10X KHK sample to each of the 4 reactions with KHK (C, D, E, &F).
- The 2x KHK Cocktail will use a mixture of pyruvate kinase and LDH. Use 8 µL so you don't have to entirely re-calculate your recipe.
- The serial dilution of Pyruvate Kinase will be a 4-fold series. See slide 25. You will make a 500-fold dilution (4 µL into 1 mL EDB) and use 133 µL in each of 3 wells of row A, then do the serial dilutions.

Announce Concepts Procedure Hazards Tips Clarification End

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
    - Table for DTNB reactions (Part I)
    - Recipes for KHK activity assay (Part II) and Coupled assay (Part III)

At the end of lab, you should have:

- Completed DTNB modification reactions (Part I) downloaded from spec.
- Completed KHK activity assays (Part II) downloaded from spec.
- Completed Coupled Assays (Part III) downloaded from spec.
- Saved and turned in to TFs the 96-well plate from DTNB reactions and any remaining quenched aliquots for Week 2
- Record columns that your TF assigned you to load your samples on the 96 well plate for Part I
- Saved all your data

Questions?

Discussion Quiz