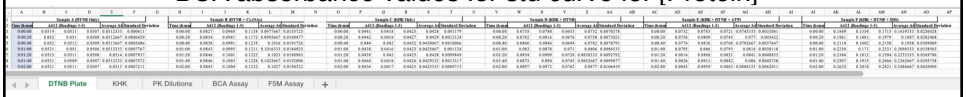
Announcements

- **Chapter 8C autoradiograph pictures should have been Slacked to each section**
- **Chapter 6/8 post-lab write-up DUE next week (March 20–25)**
- **Chapter 9 Laboratory Report Template will be up this week**
- **Start Chapter 9 report by making worksheet with 5 tabs***
  - DTNB kinetics
  - **KHK activity kinetic data**
  - PK activity kinetic data
  - F5M fluorescence values for std curve and assay
  - BCA absorbance values for std curve for [Protein]



*All except KHK activity (**bold**) should have Ave and Standard deviation calculated. This will be graded as part of Chapter 9 Laboratory Report.
You can use the worksheet to make tables and graphs for pasting into your Report.

Announce | Concepts | Procedure | Hazards | Tips | Clarification | End

Chapter 9B: Protein Modification

Objectives

- To continue learning about the enzyme ketohexokinase (KHK)
- To modify sulfhydryl group (thiol group) of cysteines residues in a protein using fluorescein-5-maleimide (F5M)
- To calculate and compare number of cysteine residues in KHK by DTNB and F5M labeling methods. For this, the [protein] in needed.

Procedures

- To use **fluorescein-5-maleimide (F5M)** to ***modify cysteines groups in KHK***
- Use **fluorescence** to determine ***amount of F5M incorporated*** into KHK
- Run BCA assay for protein-concentration determination

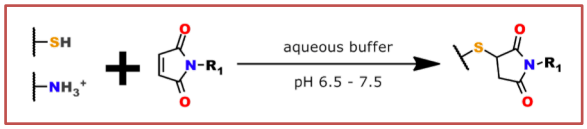
Announce | Concepts | Procedure | Hazards | Tips | Clarification | End

Labeling of Cys residues with maleimide

Thiol-maleimide reaction: Michael addition reaction

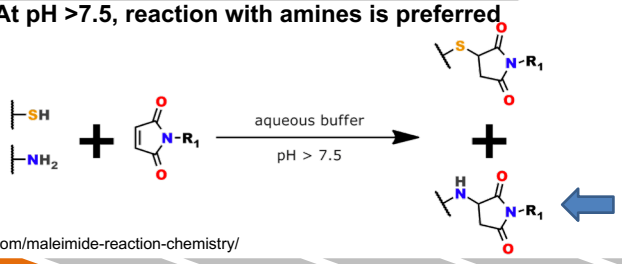
pH 6.5 to pH 7.5, this reaction is chemoselective for thiols.
At pH 7.0, reaction with thiols is ~1,000x faster than with amines (a).

(a)



At pH >7.5, reaction with amines is preferred

(b)



https://www.quantabiodesign.com/maleimide-reaction-chemistry/

Fluorescence spectroscopy

$M + h\nu \rightarrow M^*$

- It then emit light at longer wavelengths at a lower energy level (emission)

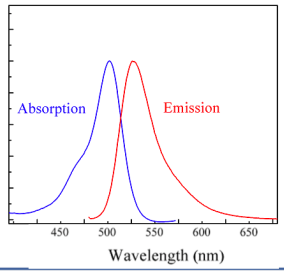
$M^* \rightarrow M + h\nu'$

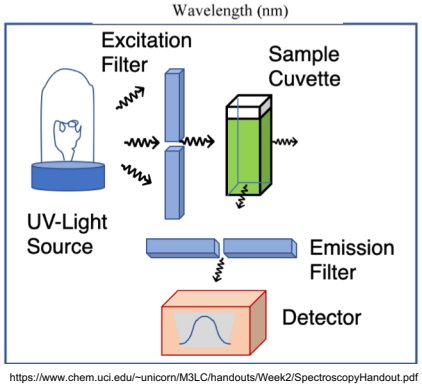
- Net reaction:

$M + h\nu \rightarrow M^* \rightarrow M + h\nu'$

$h\nu > h\nu'$

RECALL:
Energy = $h\nu = hc/\lambda$
[h is Plank's constant (J•s), ν is frequency (s^{-1}), λ is wavelength (m), c is speed of light ($m \bullet s^{-1}$)

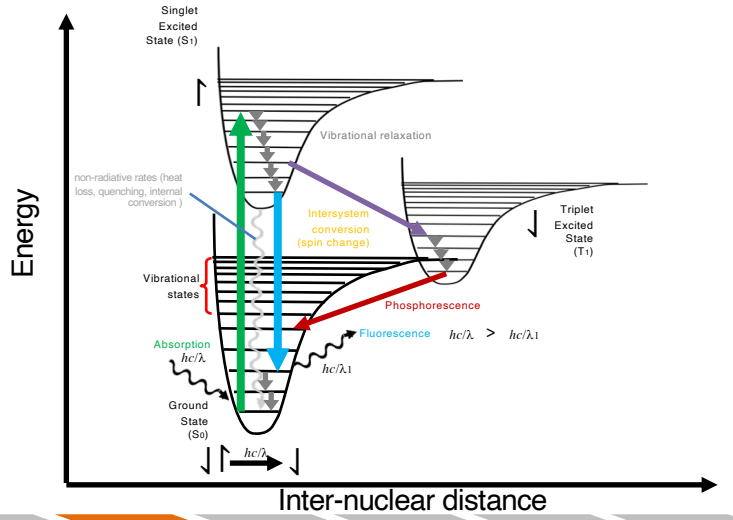




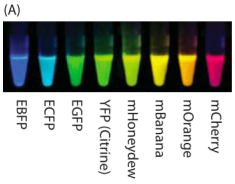
https://www.chem.uci.edu/~unicorn/M3LC/handouts/Week2/SpectroscopyHandout.pdf

Fluorescence spectroscopy

- Molecules that are capable of fluorescence are known as fluorophores
- Fluorophores absorb light energy at particular wavelengths and energy levels (absorption) and reach an excited state (excitation)



Molecules and materials can be intrinsically fluorescent or synthesized



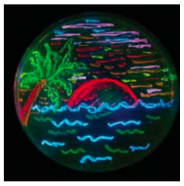


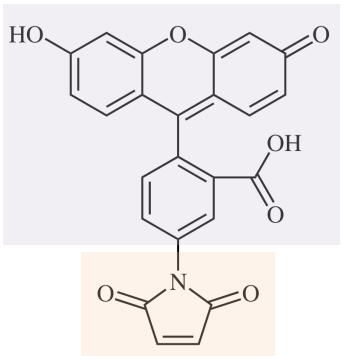
Illustration of some of the palette of fluorescent proteins that has revolutionized cell biology.
(A) Fluorescent proteins spanning a range of excitation and emission wavelengths. (B) Illustration of a petri dish with bacteria harboring eight different colors of fluorescent protein and used to "paint" an idyllic beach scene. (Adapted from: R. Y. Tsien, Nobel lecture, Integr. Biol.,2, 77-93, (2010).)

Examples of fluorescent molecules

- Amino acids (Trp, Phe, Tyr)
- Base pair derivatives (2-AP, 3-MI, 6-MI, 6-MAP, pyrrolo-C, tC)
- Chlorophylls
- Fluorescent Proteins (FPs)
- Organic dyes (fluorescein, rhodamine, N-aminocoumarins and derivatives of these)
- Rare earth elements (lanthanides)
- Quantum dots
- And many more…

Fluorescein-5-maleimide (F5M)

Fluorescein



Maleimide

- **Excitation wavelength: 494 nm**
- **Emission wavelength: 518 nm**
- Extinction Coefficient: ≥ 80,000 M^{-1} cm^{-1}
- Reactive groups: Maleimide, reacts with sulfhydryls at pH 6.5 to 7.5
- Unlike absorbance, fluorescence detection is not an absolute measurement
- Signal is usually relative to other measurements or to a reference measurement taken by an instrument

  → **Relative Fluorescent Units (RFU)**

Convert those RFUs to moles of reacted F5M

Divide the moles of reacted F5M by moles of KHK will get the number of sulfhydryl groups per protein

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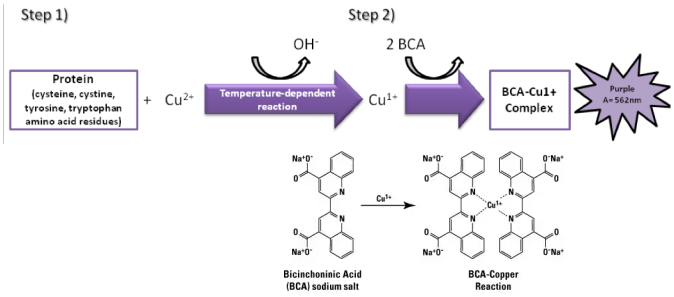
Bicinchoninic acid assay (BCA) assay

- Biochemical assay for determining the total concentration of protein in a solution (linear working range: 1-1000 µg/mL)
- Unlike methods like Bradford, the BCA Assay is compatible with samples that contain up to 5% surfactants (such as SDS)
- Color change from **green to purple in proportion to protein concentration**, quantified at **562 nm** with a spectrophotometer
- Highly alkaline solution with a pH 11.25
- Involves an incubation time (0.5–4 h at 37–60 °C)



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Two methods of protein labeling

- Method 1: Modify cysteines groups in KHK by **DTNB**
- Method 2: Modify cysteines groups in KHK by **F5M**
- To calculate and compare number of cysteine residues in KHK by these two different labeling methods
- **Do they agree with each other or not?**

- Are the values statistically different?
- Example:
  - DTNB gives you a value of 10 ± 4 cysteines
  - F5M gives you a value of 6 ± 3 cysteines

  Are there 10 or are there 6 cysteines in KHK?

General overview of Chapter 9BE

Part I: Labeling KHK with F5M

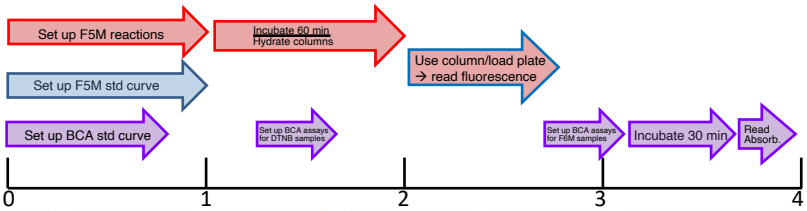
- Modify exposed Cys residues on KHK with F5M
- Use denaturants (SDS) to expose all Cys, including buried residues
  - *Would you expect a difference in fluorescence readings between these? Why?*

Part II: Standard curve with various concentrations of F5M

- Create a standard curve of RFU versus moles of fluorescein.
- Use the standard curve to relate your measured RFU to moles of Cys residues in KHK

Part III: BCA Assay

- Create a standard curve for [protein] and samples from DTNB, F5M reactions, to get number of Cys residues/KHK subunit.



Chapter 9B: Procedure

First Hour

Part I: F5M Modification Reactions

- Using **0.2 mL PCR tubes**, set up FOUR 35-µL reactions.
- Keep away from light by wrapping tubes with a layer of aluminum foil / use 0.5 mL amber tubes to set up reactions (if available)

A: KHK + F5M

B: KHK + F5M + SDS

C: F5M only (negative control)

D: KHK only (negative control)

Preparation of 35-µL reactions for KHK and F5M labeling experiments.

	HEPES buffer	KHK	F5M	SDS	H_2O	Final Volume (µL)
Fold dilution from stock	17.5x	4.6x	1.6x	10x	–	
[Stock]	350 mM	8 mg/mL	2 mM	10%	–	
[Final] in Assay	20 mM	*1.74 mg/mL	1.25 mM	1%	–	
A	2 µL	7.6 µL	21.9 µL	–	3.5 µL	35
B	2	7.6	21.9	3.5	–	35
C	2	-	21.9	–	11.1	35
D	2	7.6	–	–	25.4	35
Order of addition:	②	④	⑤	③	①	

*Note: this concentration is ~55 µM, so the F5M is about 23X molar excess of KHK

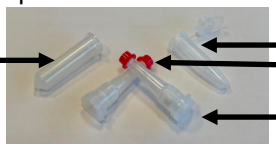
- Incubate tubes for 1 h at 37°C in a water bath

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

Chapter 9B: Procedure

Second Hour

- While tubes are incubating, obtain THREE Centri-spin10 column and hydrate the gel
  - ➢ *Why is there a need to remove the non-reacted fluorescein?*
- Gently tap the dry gel in the column so that it settles to the bottom
- Remove the top cap and add **0.65 mL of 20 mM HEPES buffer pH 7**
- Replace the cap and shake/vortex to hydrate the gel in the column fully
- Remove any bubbles by tapping or shaking
- **Allow the hydration to occur for at least 30 min at RT***
- Remove the top cap (white) and end stopper (red) of the column containing the hydrated gel and place it in a 2-mL wash tube**
- Spin column in centrifuge at **750 x g for 2 mins** (*No samples added yet!*)
- Blot any remaining drops at the end of the column with a Kimwipe



*Can start Part III

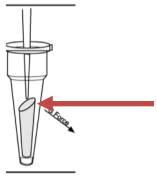
**Can be started when your F5M-incubated samples are cooling down to RT.

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

Chapter 9B: Procedure

Third Hour

- Discard the wash tube and fluid in the tube
- Once samples have cooled to RT (~5 min), briefly spin down with a benchtop mini centrifuge for ~30 s
- Hold the column up to the light and add all the 35 µL sample directly onto the center of the gel bed (*Why?* See diagram below) (*Be careful not to disturb the gel!*) KHK only sample (D) does not need to be used with column
- Place column into a **NEW 1.5-mL sample collection tube**
- Place column and tube into rotor with the highest point of the gel media pointing toward the outside of the rotor
- Spin column in centrifuge at **750 x g for 2 mins**
- Record final volume of sample in the collection tube (use P200 pipetman)



Highest point of the gel media

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

Chapter 9B: Procedure

Third Hour

- Obtain a 96-well **black** bottom plate (1/group) *Why?*
  - ➢ *Reduce background interference and cross-talk; only the light emitted from your sample will be measured*

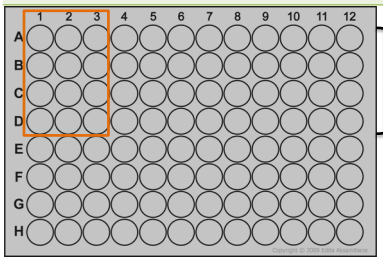
- Aliquot 90 µL 20 mM HEPES buffer pH 7 into each of the 12 wells Rows A to D, columns 1 to 3

A: KHK + F5M

B: KHK + F5M + SDS

C: F5M only (negative control)

D: KHK only (negative control)



90 µL buffer + 10 µL sample

- Remove **10 µL*** from the collection tube and aliquot it into the respective well for the sample in column 1
- Repeat the same in columns 2 and 3 for triplicate readings
- Careful, you only have ~30-40 µL!

*provided you have at least 30 µL after column

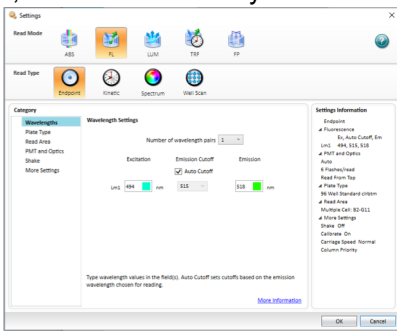
Announce > Concepts > Procedure > Hazards > Tips > Clarification > End

Chapter 9B: Procedure

Third Hour

- Open the **SoftMax Pro** software on the computer
- The plate readers will be set in the **FL (fluorescence) read mode** and **Endpoint read type** with **excitation wavelength of 494 nm**, and **emission wavelength of 518 nm** with **auto cutoff selected**
- Select the appropriate plate area containing samples for reading
- Under **Shake**, tick the box that says before first read for 3 s

Plate type: Remember that now you are using a **black bottom/opaque plate!**



Unlike doing a kinetic measurement in 9A, we will do endpoint readings in 9B → Entire process should be fast, < 5 min

- Check that there are no bubbles before clicking on the **READ** button
- Once the run has completed, remember to export and save the file in .xls

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

Chapter 9B: Procedure

First Hour

Part II: Fluorescence Standard Curve with various concentrations of F5M

- Fluorescence is not an absolute measurement
- Plate readers measure the light signal emitted by a sample in Relative Fluorescent Units (RFU)
- Need a standard curve to convert RFU to moles of thiol groups
- Please **use the same plate reader as Part I**!
- Carry out EIGHT 2-fold serial dilutions of F5M in 1.5 mL microcentrifuge tubes. Sample #9 is your background sample.
- Keep dilutions in the dark by wrapping 1.5 mL tubes with aluminum foil or, if available, use black/amber 1.5 mL microcentrifuge tubes

Preparation of Diluted F5M Standards

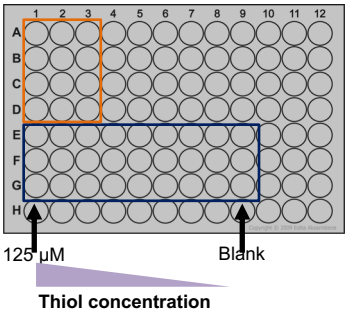
Standard #	Final thiol concentration (µM)	Volume and source of F5M	Volume of 20 mM HEPES buffer (µL)
1	125.00	100 µL 1 mM F5M	700
2	62.50	400 µL from #1	400
3	31.25	400 µL from #2	400
4	15.63	400 µL from #3	400
5	7.81	400 µL from #4	400
6	3.91	400 µL from #5	400
7	1.95	400 µL from #6	400
8	0.98	400 µL from #7	400
9	0	0	400

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

Chapter 9B: Procedure

First-Third Hour

- With a total of 8-standard and 1-background samples we want triplicate readings
  - ➢ Use rows columns 1 to 9 for the samples & rows E to G for the triplicates
- Aliquot 100 µL of sample into each well
- This part can be done prior to incubation of the F5M samples by one person while the other gets the Part I reactions started, or together right after Part I reactions are started, before equilibrating your columns
- Once yourF5M from Part I reactions are loaded, take plate with both sets of samples to the plate reader. . . . . (see prior slides)



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

Chapter 9E: Procedure

First Hour

Part III: Quantification of protein samples from 9A and 9B using BCA assay

- Make a **standard curve for your BCA assay** using different amounts of BSA (8 standards)
- Attain an aliquot (0.4 mL) of BSA stock solution (1 mg/mL)
- Use Buffer only for the Blank
- Prepare samples A-H in 1.5 mL microcentrifuge tubes

Preparation of diluted BSA standards

	Volume of previous source	Source	Volume 20 mM HEPES Buffer	Final [BSA] (µg/mL)	Final Volume (µL)
A	400	–	0	1000	200
B	200	A	200	500	200
C	200	B	200	250	200
D	200	C	200	125	350
E	50	D	200	25	200
F	50	E	200	5	200
G	50	F	200	1	200
H	50	G	50	0.5	100

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

Chapter 9E: Procedure

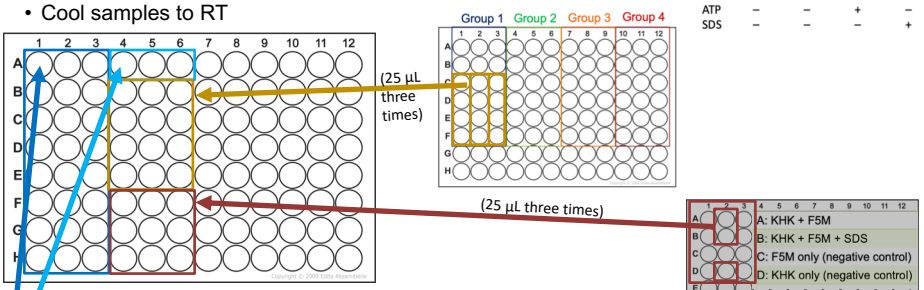
Second-Fourth Hour

Part III: Quantification of protein samples from 9A & B using BCA assay

- Obtain a new clear bottom 96-well plate (1/group)
- Aliquot 25 µL of each sample/standard to the plate. Ensure there are triplicate readings for each sample
- Add 200 µL of **BCA working reagent (WR)** to each well A1-H6
- Cover and Mix well
- Incubate tubes for 30 min at 37 °C in an oven
- Cool samples to RT

You have four samples of KHK from Part I

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



A to H (column 1- 3): triplicate of BSA standards A to H

A (column 4 - 6): triplicate of Blank

B to E (column 4 - 6): triplicate of DTNB samples

F to H (column 4 - 6): triplicate of F5M samples

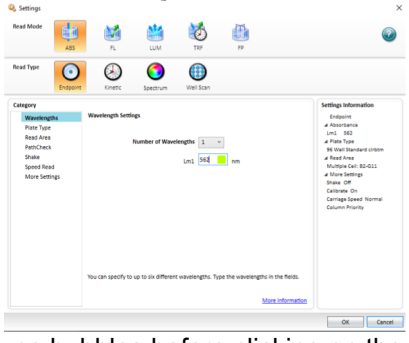
Announce > Concepts > Procedure > Hazards > Tips > Clarification > End

Chapter 9E: Procedure

Fourth Hour

- Open the **SoftMax Pro** software on the computer
- The plate readers will be set in the **ABS (absorbance) read mode** and **Endpoint read type** with **wavelength of 562 nm**
- Select the appropriate plate area containing samples for reading
- Under **Shake**, tick the box that says before first read for 3 s

Plate type: Remember that now you are using a **clear bottom plate!**



- Check that there are no bubbles before clicking on the **READ** button
- Once the run has completed, remember to export and save the file in .xls

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

CH 9BE Hazards

- Leftover buffers, and all F5M assay materials go into the mainstream waste (large carboy in the fume hood)
- BCA working reagent contains bicinchoninic acid, which is a skin irritant (ensure you wear gloves when doing your BCA assay)

Chapter 9BE Lab Tips

- Protect your fluorescence probe/samples as best as you can by wrapping it with aluminum foil (or use black microcentrifuge tubes if available)
- Pipetting very small volumes so always check to make sure you are doing it right, centrifuge briefly if necessary
- During one hour incubation time…
  - Prepare Centri-spin 10 columns (at least 30 min hydration)
  - Finish preparing and loading samples for F5M standard curve
  - Finish preparing and loading samples for BCA standard curve and DTNB samples
- When adding samples to the Centri-spin 10 columns, add directly to center of the gel bed without disturbing or touching the gel
- Save your data!

DO NOT wait until the night before or the last minute to perform error propagation calculations. These are time-intensive calculations!

Chapter 9BE Clarifications

- The KHK aliquots are 30 µL, which is just enough (7.6x3=23) for your experiment. There are no more extra aliquots, the class used the whole prep.

- The BCA assay protocol Part E, pages 302-303, has changed from the manual. Still doing same samples, but the reaction will be done in the plate rather than in separate microcentrifuge tubes; and, using the same protocol rather than different dilutions:
  - Standard curve starts with 1000 µg/mL final [BSA] and goes down to the same 0.5 µg/mL
  - 25 µL of protein solutions + 200 µL of BCA working reagent in the plate.
  - Mix and incubate whole plate at 37 ˚C for 30 min.
  - Figure 9-17 and instructions on page 304 have NOT changed

Chapter 9BE

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on GradeScope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:
    - ✓ Table for sample preparation for F5M-labeling experiment and F5M standard curve
    - ✓ Table for preparation of BSA standards for BCA standard curve
    - ✓ 96-well plate loading scheme of BCA samples

At the end of lab, you should have:

- ✓ Set up F5M reactions for KHK and negative control
- ✓ Measured fluorescence from your F5M reactions and F5M standard curve
- ✓ Carry out the BCA assay, measure absorbance, save your data
- ✓ Save your data in .xls format

Questions?

In-class activity
&
Discussion Quiz

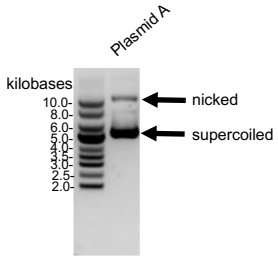


Figure 1. Agarose gel electrophoresis (1% agarose) of Plasmid A extracted by mini-prep using a QIAgen spin column and protocol. Lane 1 contains the supercoiled DNA ladder (NEB #N0472) while lane 2 contains the extracted Plasmid A sample. The gel was ran at 110 V for 1 hour before being visualized with the Gel Doc Scanner (Bio-Rad Laboratories). The strongest band observed suggests that Plasmid A is about 5-6 kb.

Problems:

1) Title or no title?
2) What is lane 1 and 2; not explicit
3) Information missing (how much loaded, how the DNA was stained, etc.)
4) Information added (last sentence is result); instead give a predicted and observed indication)
5) Use of jargon like “ran” and “extracted”

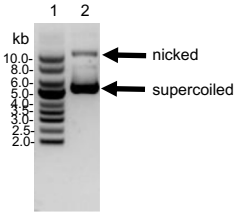


Figure 1. Plasmid Preparation Yields Mostly Super-coiled DNA. Depicted is a photograph taken with the Gel Doc Scanner (Bio-Rad Laboratories) of an agarose gel (1%) following electrophoresis at 110 V for 1 h and stained with 0.1% ethidium bromide. Lane 1 contains the supercoiled DNA ladder (NEB #N0472) (5 µL). Lane 2 contains Plasmid A preparation (2 µL) using a spin column protocol (QIAgen). The sizes of the DNA fragments from the DNA ladder are indicated to the left in kilobase pairs (kb). The 5.0 kb band represents 200 ng. The expected sizes of supercoiled (5.6 kb) and nicked-circular DNA plasmids are indicated to the right.