Isolation and analysis of proteins from red and white blood cells

Objectives

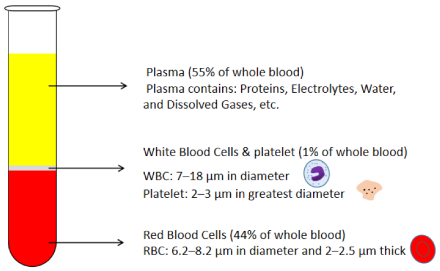
- In **week 1**, we will lyse red blood cells (RBCs) and white blood cells (WBCs) from sheep and *separate cytosolic from membrane proteins* using centrifugation.
- In **week 2**, we will conduct an assay to determine *total protein concentration* in the RBC and WBC fractions from week 1. We will also carry out the Drabkin's assay to determine the *concentration of hemoglobin* in these fractions.
- In **week 3**, we will run an *SDS-PAGE gel to visualize proteins* in the RBC and WBC fractions.

Background

Protein isolation:

In the first week of this multi-week lab, we will extract cytosolic and membrane proteins from sheep RBCs and WBCs. WBCs are nucleated cells that perform a wide range of functions in the immune system. As such, they contain many different types of proteins in the cytosol and membrane. RBCs, by contrast, lack nuclei, and play a rather singular role – the transport of oxygen in the blood. Because the RBC has such a limited function, the cytosol contains almost exclusively hemoglobin (Hb), the protein responsible for solubilizing and carrying oxygen in blood. Hb is a tetramer of four globin chains, each of which has a molecular mass of approximately 16 kDa (Dalton = grams/mole).

The starting material for this laboratory is whole blood. Whole blood is made up of an aqueous component (the plasma), RBCs and WBCs. As you can see below, WBCs make up only about 1% of the volume of whole blood.



Before lab, WBCs were purified from whole blood to save you time. To separate the WBCs from the RBCs, whole blood was placed in a hypertonic solution that lysed the RBCs. This mixture was then centrifuged to collect intact WBCs for your experiments. You will lyse these WBCs and separate the cytosolic and membrane fractions by centrifugation.

To study RBC proteins, you will be provided with a solution that already has the WBCs removed. You will lyse these cells to separate the cytosolic and membrane fractions by centrifugation.

It is important to keep the RBC and WBC reagents straight. If you mix them up, your experiments will not work properly.

Assay to determine total protein concentration and standard curves

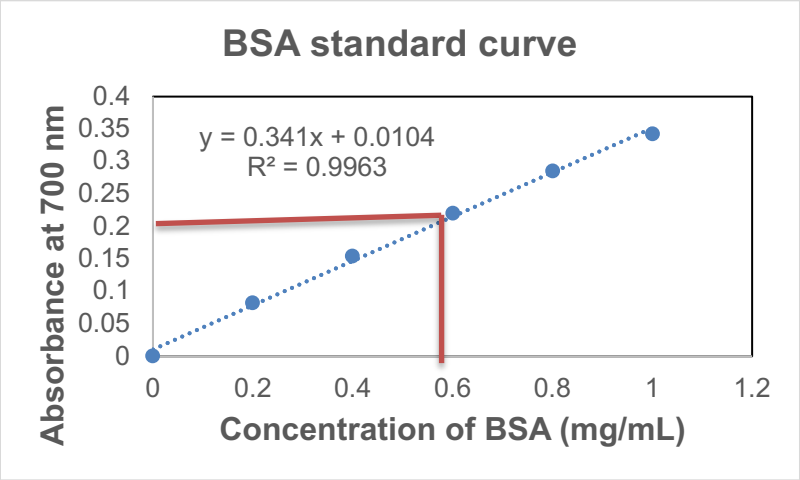
There are many different methods available to quantify proteins. The method we will utilize is the Lowry assay which was chosen over others because the reagents do not react significantly with the detergents used in the extraction solutions. This colorimetric assay is based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent (sodium 1,2-naphthoquinone-4-sulfonate). There are two steps which lead to color development: the reaction between the protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cysteine and histidine. The reduced Folin's reagent has a characteristic blue color with maximum absorbance at 700 nm. The more protein in the sample, the more blue the reaction.

Standard curves are used for the quantitative analysis of unknown substances by comparison to a known "standard". In evaluating the protein concentration of a sample, the amount in a given volume of unknown sample can be determined by comparison to the amounts of a "standard protein". A common standard in protein assays is bovine serum albumin (BSA).

Determining the concentration of protein in your RBC and WBC fractions is a three-step process:

1) Generate a standard curve using known amounts of BSA (should be linear).
2) Determine how much protein is present in a particular dilution of your unknown sample using the standard curve.
3) Calculate the concentration of protein in the unknown sample by correcting for the dilution factor.

This concept is illustrated below:



Calculation for the unknown protein sample using equation of the line:

Absorbance of unknown protein sample (1:50 diluted) = 0.2 (y)

Calculate concentration of protein in 1:50 diluted sample by solving for x:

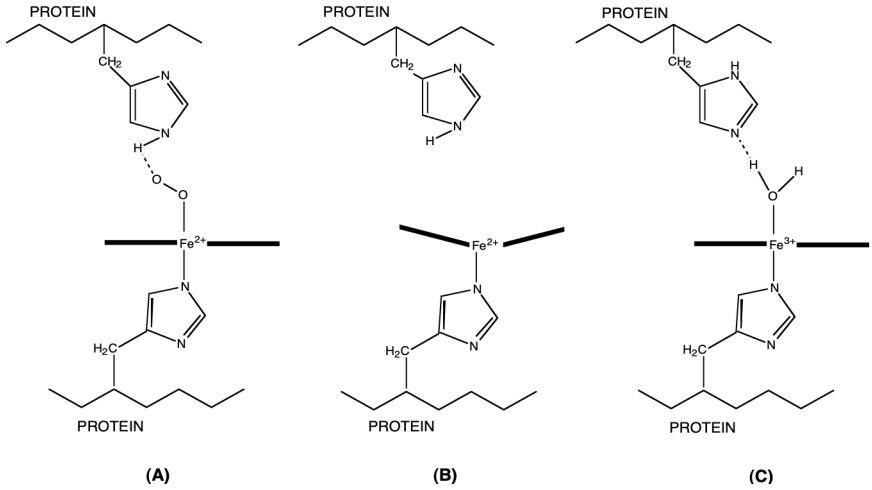
x = (y-0.0104)/0.341 = (0.2-0.0104)/0.341 = 0.56 mg/mL

Amount of protein in *undiluted* sample = 0.56 mg/mL X 50 = 28 mg/mL

We have to assay different dilutions of our unknown in order to ensure that we obtain an absorbance that lies within the linear range of the standard curve (for accuracy). If a sample is too concentrated, it would give an absorbance reading that is off the standard curve (>0.4 in this example). On the other hand, if it is too dilute, it would give an absorbance reading that is too low to be useful (<0.1 in this example).

Assay to determine hemoglobin concentration

The method we will use in this lab was first described by Drabkin and Austin in 1932. In the 1950's, several different methods were used to standardize the hemoglobin concentration in blood, and each gave very different results. In 1966, it was generally agreed that the method of Drabkin yielded the most accurate results. Drabkin's reagent converts hemoglobin from the ferrous (+2) to the ferric (+3) state to produce methemoglobin (see Met-Hb below).

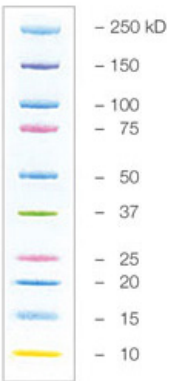


Oxy-Hb (Fe^{2+}) Deoxy-Hb (Fe^{2+}) Met-Hb (Fe^{3+})

The methemoglobin then combines with potassium cyanide (a component of Drabkin's reagent) to form cyanmethemoglobin, a stable pigment that absorbs light at 540 nm. To estimate the concentration of Hb in your RBC and WBC fractions, you will again construct a standard curve, this time using pure Hb as the standard.

SDS-polyacrylamide gel electrophoresis (denaturing conditions)

SDS-PAGE is an electrophoresis method that allows separation of proteins by mass. The matrix is a polyacrylamide-based gel (we will use pre-made gels). These gels also contain sodium dodecyl sulfate (SDS) which acts as a surfactant to mask the proteins' intrinsic charge and confers on them very similar charge-to-mass ratios. Upon application of a constant electric field, the proteins migrate towards the anode (positive electrode), each with a different speed, depending on its mass. Smaller proteins migrate through the gel faster than larger proteins thereby enabling protein separation by mass. Pre-stained "Kaleidograph" standard molecular mass markers (shown on the right) run on the same gel will allow us to determine the molecular masses of unknown proteins in the WBC and RBC fractions.



Week I, Part A: Extraction of cytosolic proteins from RBCs

Equipment and reagents

- RBCs (2 eppendorf tubes with 400 µL each)
- Phosphate buffered saline (PBS)
- RBC lysis buffer (with protease inhibitor)
- RIPA membrane solubilization buffer (with protease inhibitor)
- Beaker for liquid waste
- Table top centrifuge
- Vortex mixer
- Ice
- Shared rocking platform

Procedure

1. Obtain 2 tubes of RBCs. To remove any contaminating serum proteins, we will carry out a wash step. Add 1 mL of PBS per tube and mix by gently pipetting up and down.

2. Centrifuge both tubes at 5200 rpm for 5 minutes.

3. Carefully remove the supernatant using P200 set at 200 µL and discard in the waste beaker provided. This will require multiple pipetting to remove all the supernatant. Keep pellet on ice.

4. Resuspend the RBC pellet in 700 µl RBC lysis buffer. Place tubes on rocking platform for 10 minutes, then chill on ice for 5 minutes.

5. Centrifuge the tubes at **full speed** for 5 minutes. The **supernatant will have the cytosolic proteins** and the **pellet will have the membrane** and other cell debris.

6. Transfer the supernatant (with RBC cytosolic proteins) from BOTH tubes to one new tube and label **"RBC supe"**. Keep on ice.

7. We will now carry out two washes on the pellets (which contains RBC membrane components) to remove contaminating cytosolic proteins, particularly hemoglobin. Resuspend pellet in both tubes in 1 mL PBS by pipetting up and down with P1000. Centrifuge the tube at top speed for 5 minutes. Carefully remove the supernatant using P200 and discard in the waste beaker provided. This will require multiple pipetting to remove all the supernatant.

8. Resuspend the pellet in both tubes again in another 1 mL PBS, centrifuge and discard supernatant as in step 7.

9. To the pellet in both tubes, add 150 µL RIPA membrane solubilization buffer, pipet up and down to mix and vortex hard. Label these tubes **"RBC membrane"** and keep on ice. We will process them further in Part C.

Week I, Part B: Extraction of cytosolic proteins from WBCs

Equipment and reagents

- WBCs (2 eppendorf tubes with 0.5 mL each)
- RIPA membrane solubilization buffer (with protease inhibitor)
- Table top centrifuge
- Vortexer
- Ice
- Shared rocking platform

Procedure

1. Obtain 2 tubes of WBCs. These were prepared from whole sheep blood by lysing and removing RBCs and washing away contaminating RBC proteins with PBS. The WBCs were then resuspended in WBC lysis buffer and stored at -20°C.

2. Place tubes on rocking platform for 10 minutes.

3. Centrifuge the tubes at **full speed** for 10 minutes. The supernatant will have the cytosolic proteins and the pellet will have the membrane and other cell debris.

4. Transfer the supernatant (which contains WBC cytosolic proteins) from BOTH tubes to a single new tube and label **"WBC supe"**. Keep on ice.

5. Resuspend the pellet in both tubes (which contains WBC membrane components) in 150 µL RIPA membrane solubilization buffer, pipet up and down to mix and vortex hard. Label these tubes **"WBC membrane"** and keep on ice. We will process them further in Part C.

Week I, Part C: Extraction of membrane proteins from RBCs and WBCs

The RBC and WBC pellets from Parts A and B contain membrane components. We will now extract proteins from the membrane using RIPA buffer that contains several different detergents (SDS, sodium deoxycholate, NP-40) that will denature the membrane and solubilize membrane proteins. We will also use two freeze-heat cycles to facilitate this process. ***It is important to keep in mind that the detergents make the solutions frothy, and the solutions will also be viscous. So be careful when you pipet.***

Equipment and reagents

- RBC pellets in RIPA buffer from Part A (2 tubes)
- WBC pellets in RIPA buffer from Part B (2 tubes)
- Table top centrifuge
- Dry ice bath (VERY COLD – do not touch with bare hands)
- Heat block at 70°C

Procedure

1. Place the tubes containing your RBC pellets and WBC pellets (these should be in RIPA buffer) in a dry ice bath for 3 minutes. Wipe off the liquid from tubes with kimwipe, then place in 70°C heat block for 3 minutes.

2. **Repeat step 1.**

3. Centrifuge the tubes for 10 minutes at **full speed.**

4. Label a fresh Eppendorf tube **"RBC membrane".** After the centrifuge is done, carefully collect the supernatant from the two RBC tubes using a P200 and combine into the one tube.

5. Label a second Eppendorf tube **"WBC membrane",** and carefully combine the supernatants from the two centrifuged WBC tubes into this tube.

6. **Give all four tubes (RBC supe, RBC membrane, WBC supe, WBC membrane) to instructor for storage at -20°C. Make sure tubes are identifiable as yours (label appropriately).**

Week 2, Part I: Lowry Assay to determine <u>total</u> protein concentration

**** Please calculate your dilutions (steps 2 and 3) as a pre-lab exercise and check with me before you start your experiment ****

<u>Equipment & reagents</u>

- Four fractions from first week (RBC supe, RBC membrane, WBC supe, WBC membrane)
- Tube of water to make dilutions
- Standard BSA solution (1 mg/mL)
- Tube of Reagent A
- Tube of Reagent B
- Glass tubes for assay
- Cuvettes
- Spectrophotometer set at 700 nm
- Vortex mixer

<u>Procedure and analysis</u>

1. Thaw your samples at room temperature and invert tubes to mix.

2. For the BSA standard curve, set up **six eppendorf tubes** and label appropriately. Add BSA standard stock solution (1 mg/mL) to obtain the final concentrations shown below. Final volume will be 100 µL. (Use $C_1V_1=C_2V_2$ to calculate V_1 of BSA stock).

Final concentration (mg/mL)	**µL of 1 mg/mL BSA to add**	**µL of water to get to 100 µL**
0	0	100
0.2		
0.4		
0.6		
0.8		
1	100	0

3. Since you have no idea how concentrated/dilute your samples are, you will need to assay different dilutions of your sample to cover a range of concentrations, to ensure that at least one dilution "hits" the linear range of the BSA protein standard curve. <u>We will try three dilutions per sample: undiluted, 1:10 diluted, 1:100 diluted.</u> **Vortex your samples** and then prepare your 1:10 and 1:100 dilutions in **eppendorf tubes in 100 µL final volume of water. Label tubes appropriately, so you don't mix them up!**

Dilution	**µL of sample to add**	**µL of water to get to 100 µL**
1:10		
1:100		

4. Vortex all tubes (for BSA standard curve and fraction dilutions) after you've prepared them. These dilutions will be used to prepare samples for Lowry assay below.

5. Now prepare **disposable glass tubes** for the Lowry assay. ***Label your tubes appropriately, so you don't mix them up!!***

a) 6 tubes for BSA standard curve (0-1 mg/mL range)
b) 3 tubes for RBC supe (undiluted, 1:10, 1:100)
c) 3 tubes for RBC membrane (undiluted, 1:10, 1:100)
d) 3 tubes for WBC supe (undiluted, 1:10, 1:100)
e) 3 tubes for WBC membrane (undiluted, 1:10, 1:100)

6. Pipet 125 µL Reagent A into each tube.

7. Add 25 µL of appropriate protein sample to appropriate tube, and **immediately vortex**.

8. Add 1 mL Reagent B to each tube, and **immediately vortex**.

9. Incubate tubes for 10 min at room temperature.

10. Pour the reactions into labeled cuvettes.

11. Set the spectrophotometer to 700 nm. **Use the 0 BSA sample to blank the spectrophotometer** and record absorbance at 700 nm for the remaining BSA standards and unknown samples.

12. Construct a BSA standard curve (mg/mL of protein on x-axis, absorbance at 700 nm on y-axis). Determine the amount of total protein in your sample by solving for x using the equation of the straight line. *(See Background for reminder on how to do this).* ***DON'T FORGET YOUR DILUTION FACTOR!!***

13. Glass tubes should be disposed in Glass Waste Carton. Everything else can be disposed of in regular trash.

Week 2, Part II: Drabkin Assay to determine hemoglobin concentration

Equipment & reagents

- Four fractions from first week (RBC supe, RBC membrane, WBC supe, WBC membrane)
- Standard hemoglobin solution (4 mg/mL)
- Tube of Drabkin Reagent
- Glass tubes for assay
- Cuvettes
- Spectrophotometer set at 540 nm
- Vortex mixer

Procedure and analysis

1. For the Hb standard curve, set up **six disposable glass tubes and label appropriately**. Add Hb standard stock solution (4 mg/mL) to obtain the final concentrations shown below. Final volume will be 1000 µL in **Drabkin's reagent**. (Use $C_1V_1=C_2V_2$ to calculate V_1 of Hb stock and *check calculations with me before you start*).

Final concentration (mg/mL)	**µL of 4 mg/mL Hb to add**	**µL of Drabkin to get to 1000 µL**
0	0	1000
0.16		
0.4		
0.8		
1.2		
1.6	400	600

3. Now prepare **eight disposable glass tubes** for your unknowns. ***Label your tubes appropriately, so you don't mix them up!!*** Add the appropriate volume of Drabkin reagent to the tubes. Then **vortex your samples** before adding to the Drabkin reagent.

Sample	**µL of sample to add**	**µL of Drabkin to get to 1000 µL**	**Dilution factor**
RBC supe	50 µL		
	10 µL		
RBC membrane	50 µL		
	10 µL		
WBC supe	50 µL		
	10 µL		
WBC membrane	50 µL		
	10 µL		

4. Vortex all tubes (for Hb standard curve and unknown fractions) after you've prepared them. Incubate for 5 minutes at room temperature, then pour reactions into labeled cuvettes.

5. Set the spectrophotometer to 540 nm. **Use the 0 Hb sample to blank the spectrophotometer** and record absorbance at 540 nm for the remaining Hb standards and unknown samples.

6. Construct a Hb standard curve (mg/mL of protein on x-axis, absorbance at 540 nm on y-axis). Determine the amount of Hb in your sample by solving for x using the equation of the straight line. *(See Background for reminder on how to do this).* ***DON'T FORGET YOUR DILUTION FACTOR!!***

7. **Return all four original tubes (RBC supe, RBC membrane, WBC supe, WBC membrane) to instructor for storage at -20°C. Make sure tubes are identifiable as yours (label appropriately).**

8. Glass tubes should be disposed in Glass Waste Carton. Everything else can be disposed of in regular trash. **Important:** please remember to turn off the spectrophotometer after you are done for the day.

Week III. Assessment of RBC and WBC protein composition by SDS-PAGE analysis.

Pre-lab exercise. Please calculate (<u>using Lowry total protein data</u>) the volume of RBC supe, RBC membrane, WBC supe, WBC membrane you need to get <u>20 µg of total protein</u>. Check calculations with me prior to starting experiment.

<u>Equipment & reagents</u>

- Four fractions from first week (RBC supe, RBC membrane, WBC supe, WBC membrane)
- Tube of water
- 4X Loading dye
- Heat block at 95°C
- Table top centrifuge
- 4-20% TGX gels
- Kaleidoscope standard markers (10-250 kDa)
- Electrophoresis chamber
- Laemmli running buffer
- One-Step Blue Protein Gel Stain

<u>Procedure and analysis</u>

Wear gloves and don't get solutions on your skin or clothes. The gel stain, in particular, will not come off.

1. For each of your samples, you should have calculated (pre-lab) what volume you need to get 20 µg of TOTAL PROTEIN.

For example if your sample = 5 µg/µL, then for 20 µg, you would need 4 µL.

If your sample is too concentrated, you will have to dilute it in water and then take the appropriate volume of the diluted sample for 20 µg. For example, if your sample is 100 µg/µL, for 20 µg, you would need 0.2 µL, which you cannot possibly pipet accurately. So, you might want to dilute the 100 µg/µL sample 10-fold to 10 µg/µL, and then take 2 µL of the diluted sample for 20 µg.

2. Pipet the appropriate volume of each sample into appropriately labeled **eppendorf tubes**, and add water to get a ***final volume of 24 µL.***
*** If your sample is too dilute to obtain 20 µg of protein, just take 24 µL of your concentrated sample* ***and calculate how much protein you have in that 24 µL volume.***

Sample	**Total protein (µg/µL)**	**µL of sample for 20 µg**	**µL of H_2O to get to 24 µL**
RBC supe			
RBC membrane			
WBC supe			
WBC membrane			

3. To each tube, add 8 µL 4X loading dye.

4. Place tubes in heat block set at 95°C for 5 minutes.

5. Centrifuge samples at full speed for 15 seconds.

6. Assemble gel running apparatus with instructor's assistance:
    a. Obtain gel from instructor
    b. Gently pull out comb and discard
    c. Peel off green strip from bottom of the gel and discard
    d. Insert gel into chamber and gently pour running buffer to completely cover gel
    e. Using the P200 set to 50 µL, gently clean out the wells with the running buffer

7. Load 10 µL of the Kaleidograph protein standards into lane 1.

8. Load entire 32 µL of each of your samples onto the gel as follows:
    a) RBC supe into Lane 3
    b) RBC membrane into Lane 5
    c) WBC supe into Lane 7
    d) WBC membrane into Lane 9

9. Run the gel at 200 V for 30-40 minutes.

10. After taking down the gel (with instructor's assistance), stain overnight in a labeled tray with One-Step Blue Protein Gel Stain (**wear gloves).**

11. The following day, instructor will photograph your gel using the gel documentation system and post it on Moodle for your analysis.

Lab report- please include all the information below in your lab report which is worth 25 points and is due Wednesday, 10/21/26, in lab. Please remember that lab reports are non-collaborative – i.e. must be written independently of your group/class mates.

1. **Total Protein Concentration Data (Lowry assay)**
A) Include as **Figure 1**, BSA standard curve from Lowry assay (with appropriate title and figure legend).
B) For your "RBC supe" sample, show how you calculated total protein concentration (in mg/mL). Remember to correct for dilution factor in your calculations.
C) Include as **Table 1** the absorbance at 700 nm you obtained in the Lowry assay for ALL your samples, the sample dilutions, and the total protein concentration.

2. **Hemoglobin Concentration Data (Drabkin's assay)**
A) Include as **Figure 2**, hemoglobin standard curve from Drabkin assay (with appropriate title and figure legend).
B) For your "RBC supe" sample, show how you calculated hemoglobin concentration (in mg/mL). Remember to correct for dilution factor in your calculations.
C). Include as **Table 2**, absorbance at 540 nm for ALL your samples, the sample dilutions, and the hemoglobin concentration.

Important note: in determining total protein/hemoglobin concentration, you should only use data that falls within the standard curves. Data outside standard curve are not reliable!

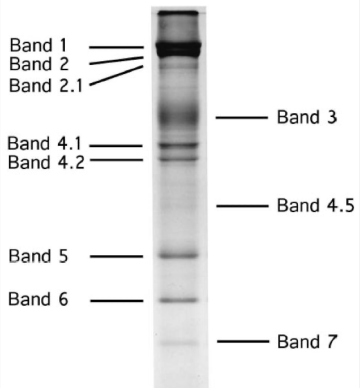
3. Construct **Table 3** as follows and fill in the information.

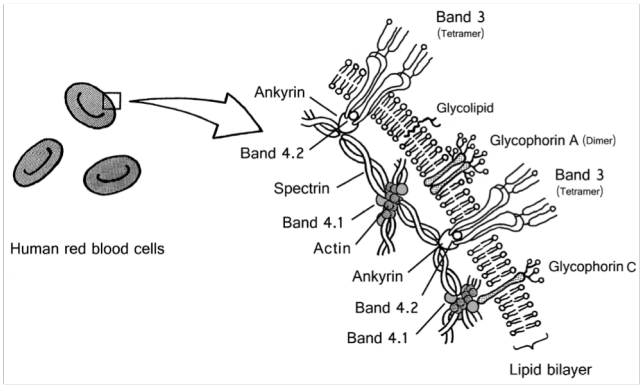
Sample	**Total protein (mg/mL)**	**Hemoglobin (mg/mL)**	**% hemoglobin in total protein**
RBC supe			
RBC membrane			
WBC supe			
WBC membrane			

Please turn over for additional information to incorporate into your lab report.

4. Include as **Figure 3** your SDS-PAGE gel, and write a figure legend to indicate what your figure is showing. Label molecular size markers (sizes in kDa). Label the lanes appropriately. Use an arrow to mark the location of the hemoglobin band and its molecular mass. Do the same (arrow and approximate molecular mass) for any other **RBC membrane proteins** you are able to identify on your gel. An SDS-PAGE gel image of RBC membrane proteins is shown below for your reference. The accompanying table identifies each protein and its molecular mass. **NOTE: if your gel does not have any visible RBC membrane proteins, please ask Dr. Chander for a sample gel to analyze.**

Band	Molecular Mass (kDa)	Designation	Function
1	240	spectrin (α chain)	components of cytoskeleton
2	220	spectrin (β chain)	
2.1	210	ankylin	
3	100	band 3 (AE1)	anion transporter
4.1	82	band 4.1	components of cytoskeleton
4.2	76	band 4.2	
4.5	55	band 4.5 (GLUT1)	glucose transporter
5	43	actin	components of cytoskeleton
6	35	glyceraldehyde-3-phoshate dehydrogenase (GAPDH)	glycolytic enzyme
7	31	stomatin	–





Data and images from Aoki, T. 2017. Membranes 7:56.

5. Using the data from Table 3 and your gel, summarize your results by addressing the following points:

- What fractions had hemoglobin? Was this expected? If not, discuss possible reasons for unexpected results.
- Was the % of hemoglobin in total protein that you calculated in Table 3 accurately reflected in the SDS-PAGE gel? If not, discuss possible reasons for unexpected results.
- Were there any other inconsistent or unexpected observations?
- Compare the "proteomes" (protein composition) of RBCs and WBCs on your gel. How many distinct protein bands can you distinguish in each of the four fractions? Can you comment (in a general sense) why you think the proteomes of RBCs and WBCs is so different? Hint: consider the biological role/function of red vs white blood cells.

This summary should be no more than 2 pages (Arial, 11 pt. font, 1.5 line spacing).

Important things to keep in mind when writing lab reports

Figures

The figure should be numbered, include a title, and include a detailed explanatory caption (figure legend) ***below*** the figure. The entire figure (along with title and legend should be on the same page). Here is an example:

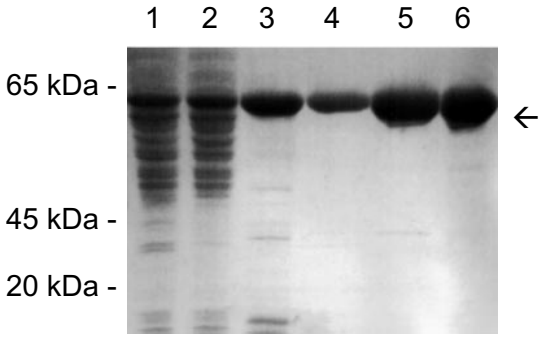


FIG. 2. SDS–PAGE of B. stearothermophilus PGM. SDS–PAGE of samples taken throughout the purification of methionyl PGM was carried out under reducing conditions as described under Materials and Methods, and the gel was stained with Coomassie blue. 5 µg of protein was run in the various lanes except for lane 4, which had only 3.5 µg. The samples in the various lanes are from (1) crude lysate supernatant fluid; (2) dialyzed ammonium sulfate precipitate; (3) heat step supernatant fluid; (4) DEAE–Sephadex column pool; (5) octyl-agarose column pool; and (6) S200 column pool. The numbers to the left of the figure indicate the position of molecular mass markers in kDa run on the same gel, and the arrow denotes the PGM.

Graphs

- A graph is also a figure and should be numbered and labeled according to the rules for Figures.
- Axes should be labeled and include units of measurement
- Tick marks on both axes should be at intervals frequent enough to allow reader to estimate value of each data point
- The meaning of each symbol should be clearly indicated
- Here is an example:

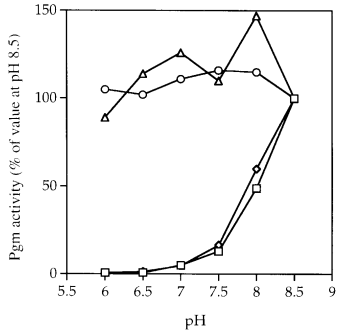


Fig. 1. The response of Pgms to pH. The Pgms from *C. perfringens* (□) and *Staphylococcus saprophyticus* (□) were treated with EDTA prior to assaying by the two-step assay with 10 µM total Mn^{2+} at various pHs. The Pgms from *M. luteus* (□) and *Streptomyces coelicolor* (Δ) were assayed at the same pHs in the absence of metals with 1 mM DPG. Pgm activity is reported as a percentage of the activity obtained at pH 8.5.

Tables

Tables should be numbered and have a short, but informative title ***above*** the table. Any details that you want to highlight can be included as a footnote to the table. The entire figure (along with title and legend should be on the same page). Here is an example:

Table 3. The pH dependence of *C. perfringens* Pgm in the presence of Co^{2+}.

pH 6.0		pH 7.0		pH 8.0	
Co^{2+} concn.	Pgm activity	Co^{2+} concn.	Pgm activity	Co^{2+} concn.	Pgm activity
None	<0.1	None	<0.2	None	<0.1
7.5 μM	2	3.2 μM	11	2.7 μM	28
77 μM	7.6	32 μM	33	28 μM	61
1 mM	11	0.4 mM	52	0.3 mM	100

Notes: Assays were carried out using EDTA-treated Pgm as described in the text. Co^{2+} concentrations given are the free concentrations. PGM activity is reported as a percentage of the activity obtained at pH 8.0 with 0.3 mM free Co^{2+}. This value was set at 100%.

Literature cited

Sources should be cited within the text where appropriate. Include full citations for any references (including textbooks, lab manuals, websites) you cite in your lab report in the *Bibliography* at the end of your lab report. This list will permit the interested reader to check the accuracy of any factual statements you make, and often, to understand the basics of your interpretations of the data. The Bibliography should cite ALL authors, date of publication, title of article, journal name, journal volume and page numbers (see below). This is in contrast to in-text citations which typically only mention the first author and date of publication (ex. Chander et al., 2021).

Format for journals:

> Cohen, F.E., and Prusiner, S.B. 1998. Pathologic conformations of prion proteins. *Annu. Rev. Biochem.* **67:** 793-819.

Format for books:

> Nelson, D.L. and Cox, M.M. 2013. *Lehninger Principles of Biochemistry*, sixth ed. Freeman and Co., NY. pp. 25-61.

Format for lab manual or handout:

> Biology 255 Laboratory Manual. 2016. Bioassay Lab, pp. 1-6. Bryn Mawr College, Bryn Mawr, PA.

Format for the web:

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