BMOL2201/6201 PRACTICAL 4

Separation of Proteins: Salting Out, Gel Filtration Chromatography and Paper Chromatography

PM	AM	Outline
2:05	10:05	Introductory talk
2:15	10:15	**(A) Salting Out** (Tutor demonstration)
2:20	10:20	(Go to iLearn and complete Pre-lab Quiz if needed)
2:25	10:25	**(B) Protein Seperation by Gel Filtration** **(C) Paper Chromatography**
4:20	12:20	Clean up
4:30	12:30	Go to iLearn for upload and Prac Quiz
4:55	12:55	Prepare to leave lab

Practical 4 Aims

- Observe **salting in and salting out** of **egg albumin protein** solution (in egg white) using **common salt** (sodium chloride; demo) *(understanding protein solubility and the role of pI for selective dissolution and precipitation).*

- Separate the protein Bovine Serum Albumin(BSA) from NADH using gel filtration chromatography (sample data provided) *(separation of nucleic acids/DNA from proteins)*

- Investigate the **reversibility of an enzyme reaction,** catalysed by Glutamic Pyruvic Transaminase (GPT), using the separation method of **paper chromatography** (chromatographs provided)*(application of separation technique to check out an enzyme reaction in metabolism).*

- *Associated Lectures for revision:*
  - *Lectures 3,4,7,8,19.*

Separation of Biological Molecules

- Most biological samples contain several different proteins.

- Proteins separation techniques include:

  A. **Salting out (Demo)**

  B. **Gel Filtration Chromatography**

     – **Follow progress of separation using spectrophotometer.**

  C. **Paper Chromatography (done for you)**

     – **Investigate the reversibility of the transamination enzyme reaction, using paper chromatography to separate the reaction mixture components.**

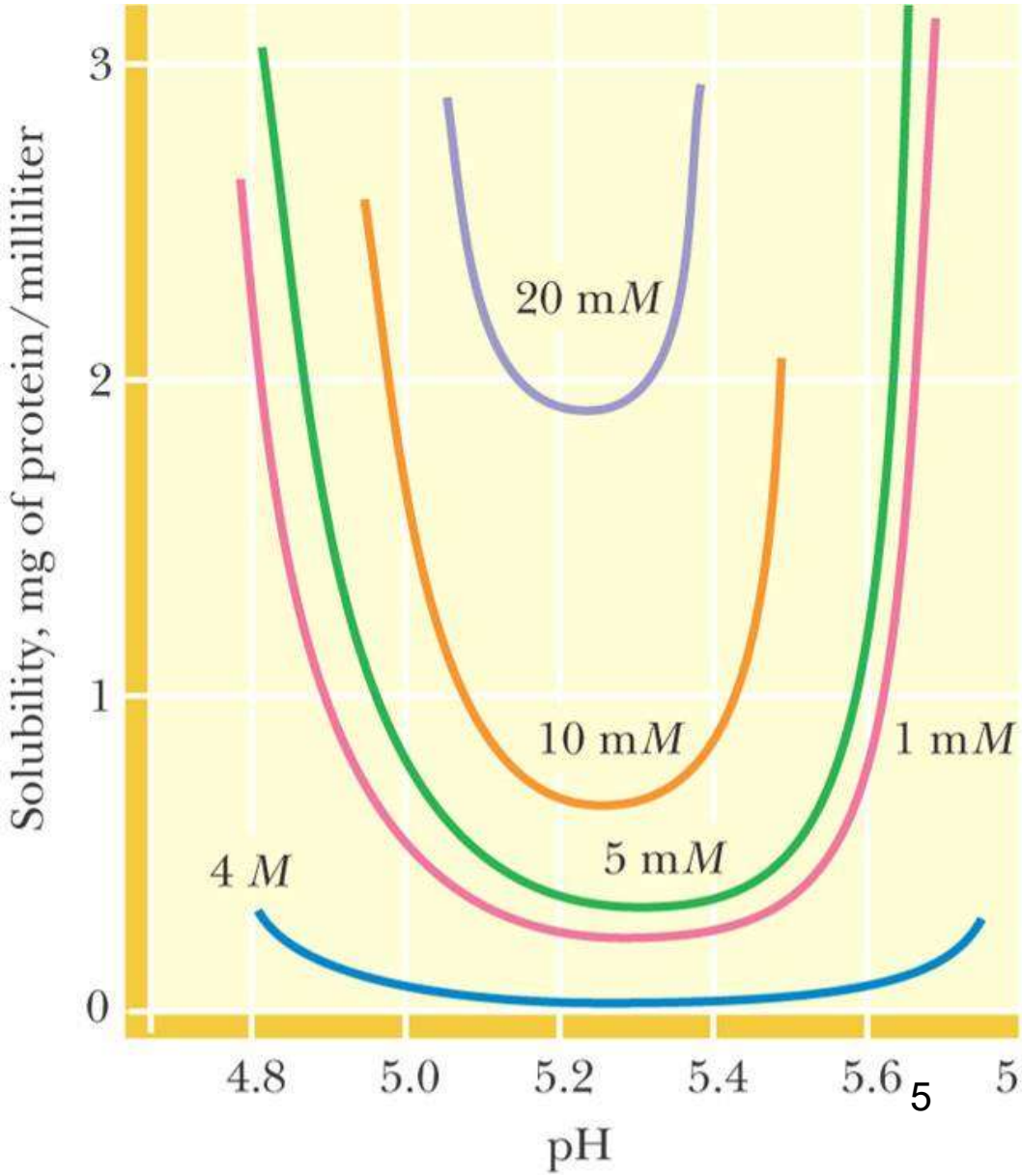
Solubility and ionic strength

- **Salting-in**
  - Most globular proteins are only slightly soluble in pure water
    - The addition of salts to increase the solubility of these proteins is called salting-in (typically ~20 mM)
    - The added salt induces charge interactions between the protein and the salt, rather than between protein molecules, thus preventing aggregation.
- **Salting-out**
  - Addition of excessive salt (>1 M) precipitates the protein
    - The salt removes all the available water and hence the protein precipitates
    - Salts with multiple charge are very effective.

Increasing ionic strength at first increases the solubility of proteins (salting-in), then decreases it (salting-out).

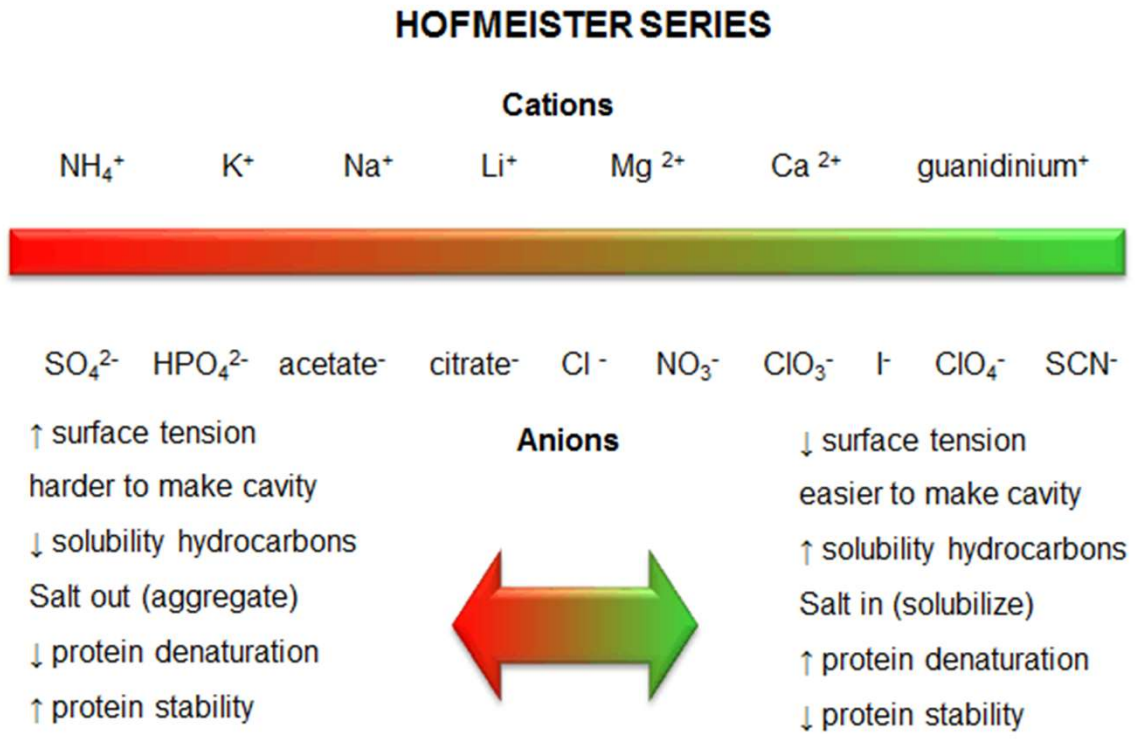
Protein solubility vs pH

- Proteins are least soluble at their **pI**, when the net charge is zero.
  - At the pI, there is little repulsion between the protein molecules; so, the protein aggregates and precipitates out.
- Generally, solubility increases with ionic strength (in the mM range).
  - The solubility of a typical protein as a function of pH and various salt concentrations.

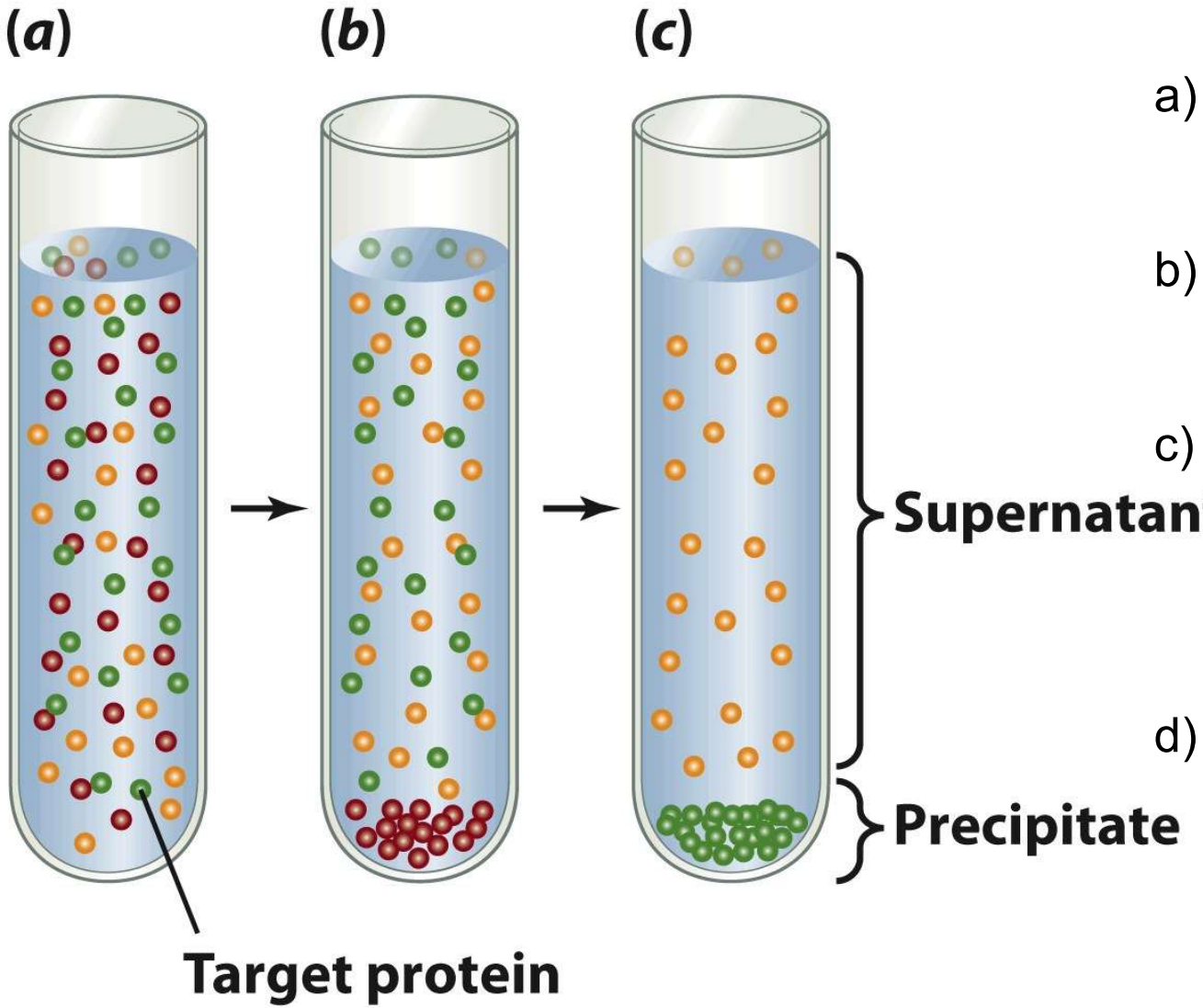


Different ions affect the solubility of proteins

- Doubly charged anions do better than singly charged



Fractionation by Salting Out



a) Add salt of choice to solution

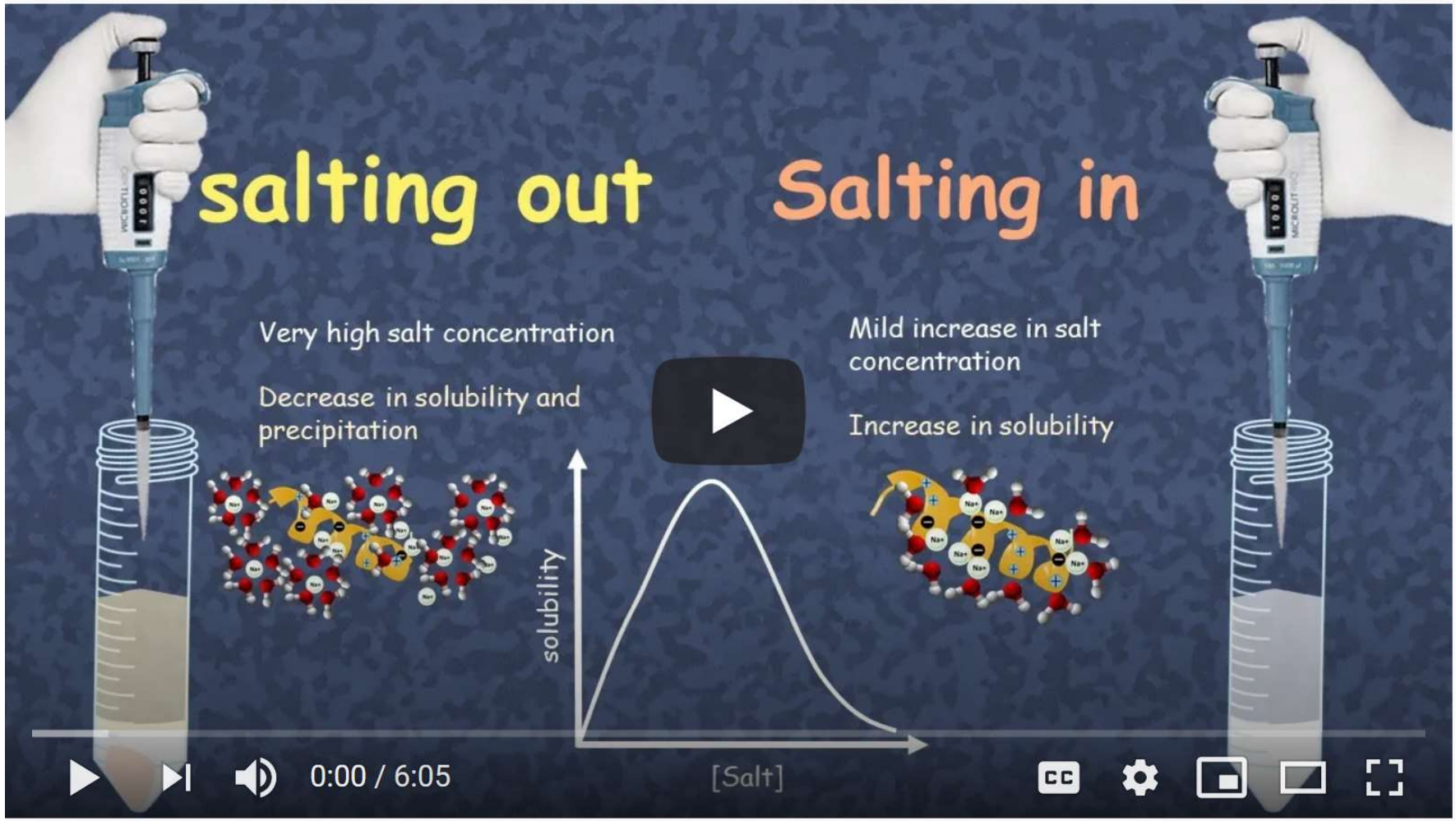
b) Centrifuge and discard unwanted proteins (red)

c) Add more salt to precipitate the protein of interest (green) and centrifuge (salting out)

d) Of course, to recover the green protein and get it into solution (salting in), we need to decrease the salt content!!!!

Video – Salting in and Salting out

- https://www.youtube.com/watch?v=gnhUh6qVD5Y

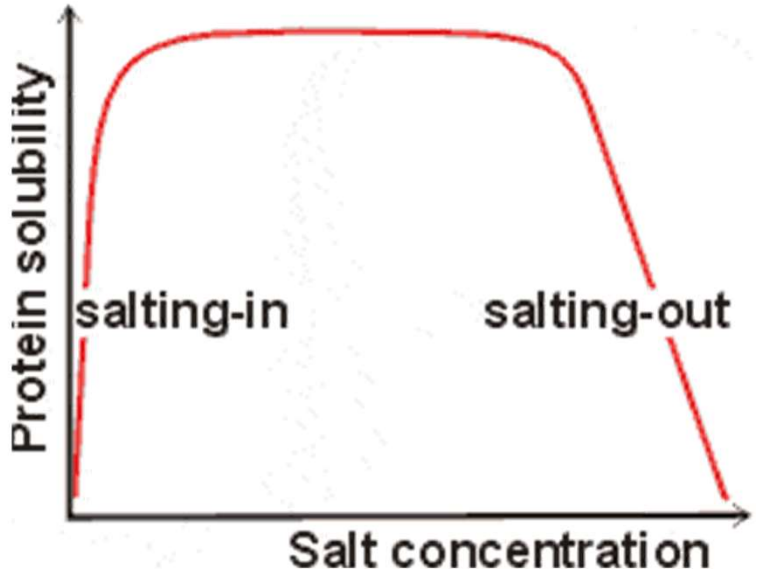


(A) Salting out of proteins (Demonstration by tutor)

Aim: Use an **egg albumin protein solution** and a saturated solution of ammonium sulfate to demonstrate **salting in and salting out.**

Method:

1. Prepare 2 tubes with 1mL egg albumin protein solution.
2. To Tube 1, add 1 mL ammonium sulfate solution.
3. To Tube 2, add 1 mL water.
4. **Protein will precipitate out in Tube 1.**
   - If not, continue adding salt (ammonium sulfate).
5. Then, **add water to each tube**, shake and check what happens.



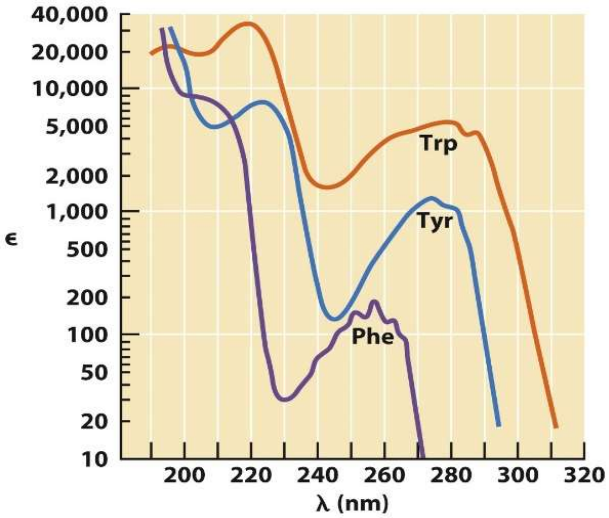
Prac 4 Excel worksheet **A. Salting out:**

1) Note your observations and answer all questions

Chromatography: “colour writing” for separation

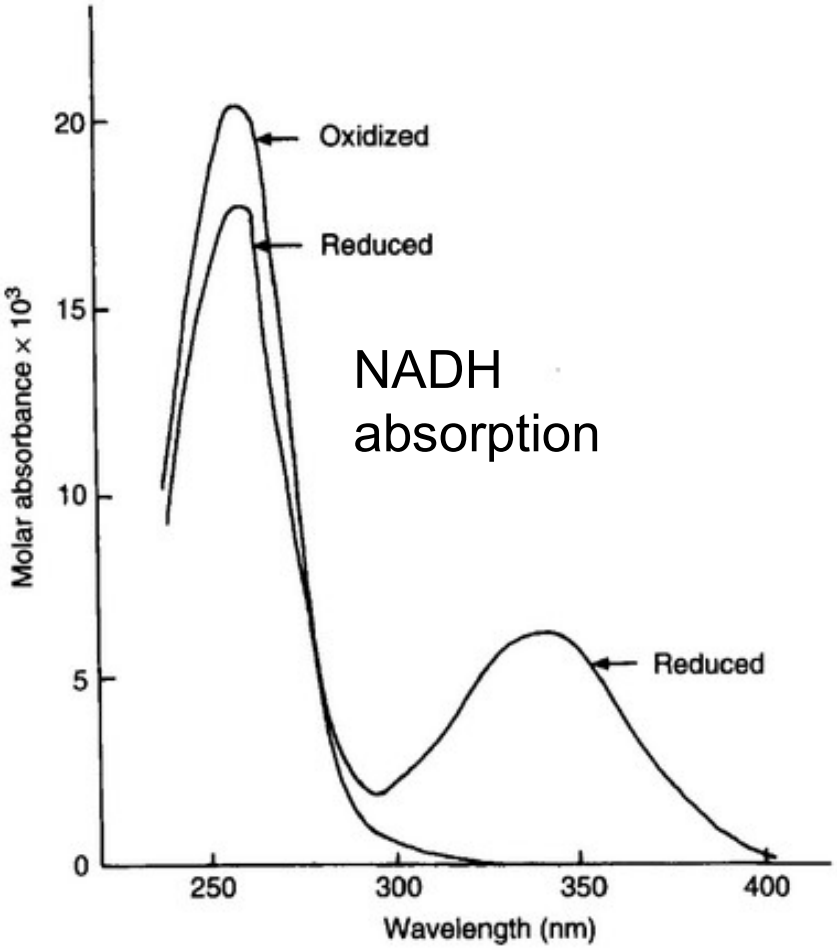
Chromatography – an analytical technique used to separate a mixture of substances, dissolved on a liquid (the “mobile” phase), based on their attraction or affinity for a solid (the “stationary” phase) – originally used to separate coloured substances.

- **Column chromatography:** Porous substances used as stationary phase (called matrix) and buffers as mobile phase. There are different types of columns depending on the nature of substances to be separated (Prac 4 Part B)
  - Presence of proteins can be monitored by collecting fractions of the mobile phase and determining the protein concentration by absorption spectrophotometry at 280 nm.
  - Plotting absorbance at 280 nm gives graph (chromatogram) with peaks for each protein separated by the column.

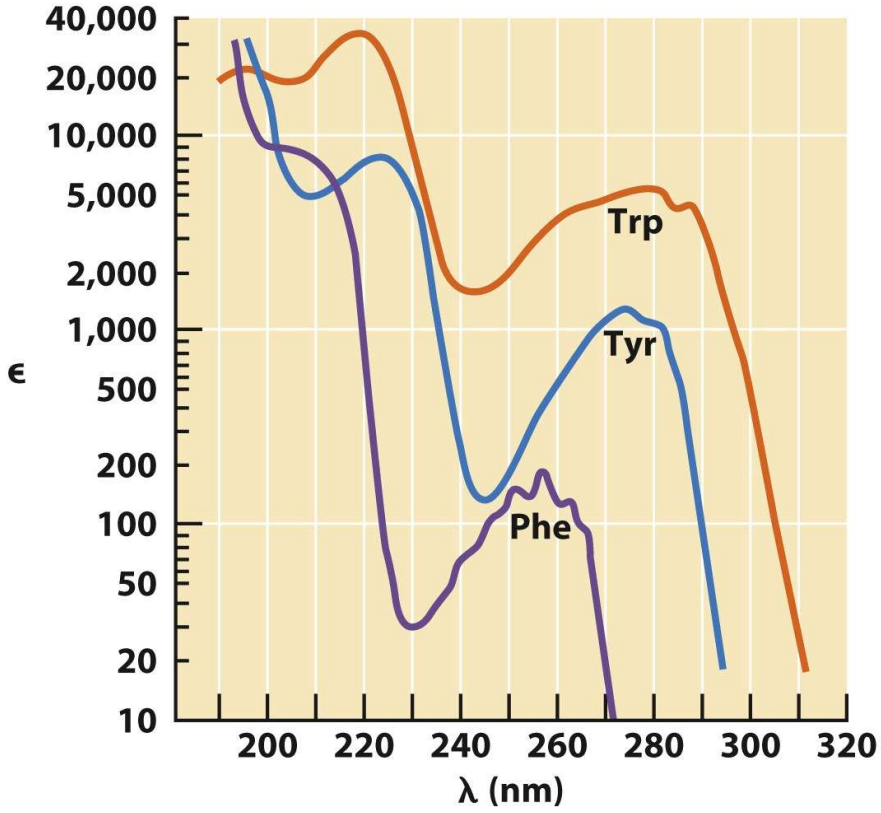


- **Paper chromatography :** Filter paper used as stationary phase and a mixture of solvent and buffer as the mobile phase (Prac 4 Part C).

Biomolecules absorb light!



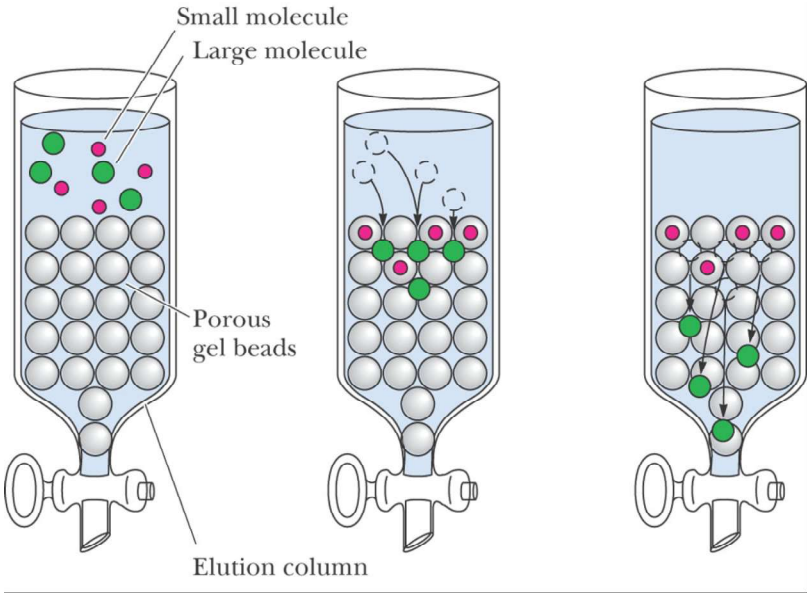
NADH absorption

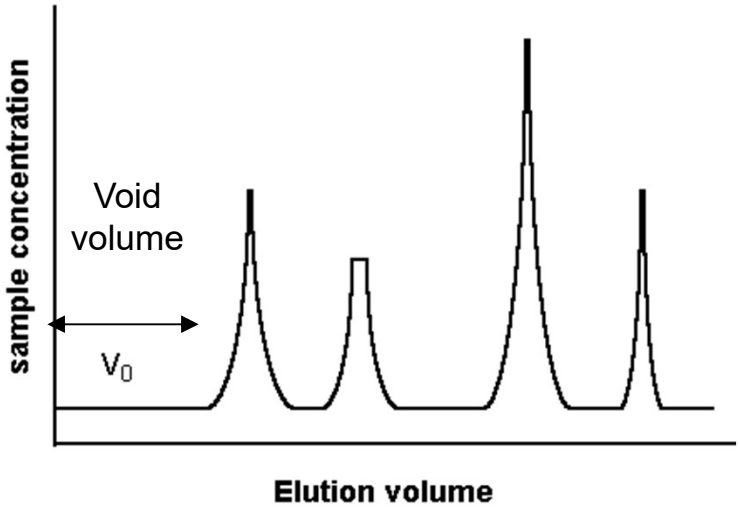


- NADH has a strong absorbance at 340 nm.
- Nucleic acids and nucleotides @ ~260 nm

Most proteins absorb light ~280 nm.

(B) Gel Filtration Chromatography





A) Aka **size exclusion chromatography**; separates proteins based on size.

B) When a solution is passed through a column packed with gel, molecules get separated.

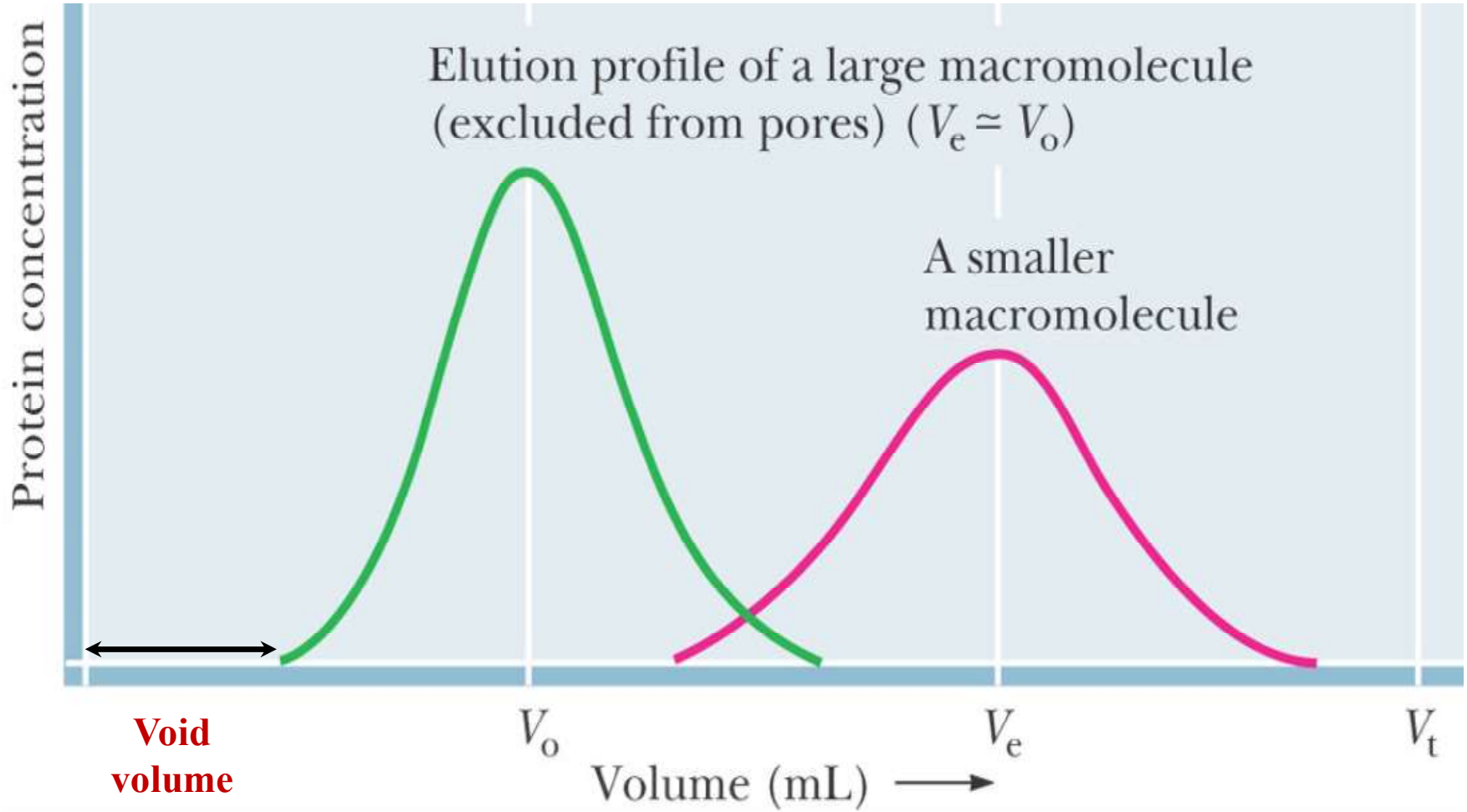
C) Small molecules diffuse into the pores, while larger molecules are excluded.

D) Large molecules exit first, with the smaller ones eluting later from the column.

E) We are going to **track** the **concentration of the solutes** by measuring the **absorbance** at wavelengths specific for proteins and nucleic acids or nucleotide cofactors.

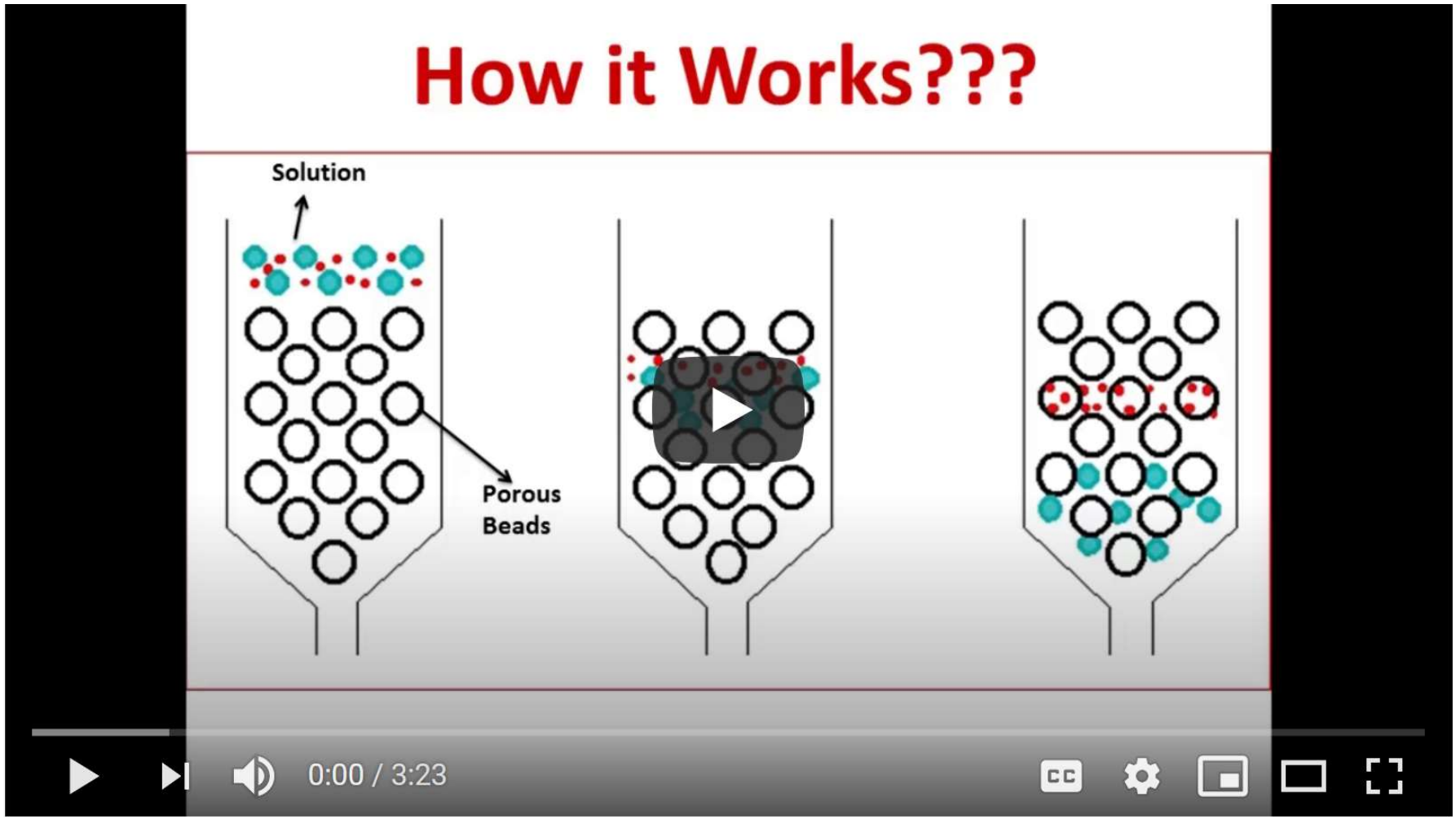
Separation of two biomolecules based on size, on a gel filtration column

- Void volume is the volume upto which A = 0



Animation- Gel filtration chromatography

- https://www.youtube.com/watch?v=ZA6rgm8wnXE

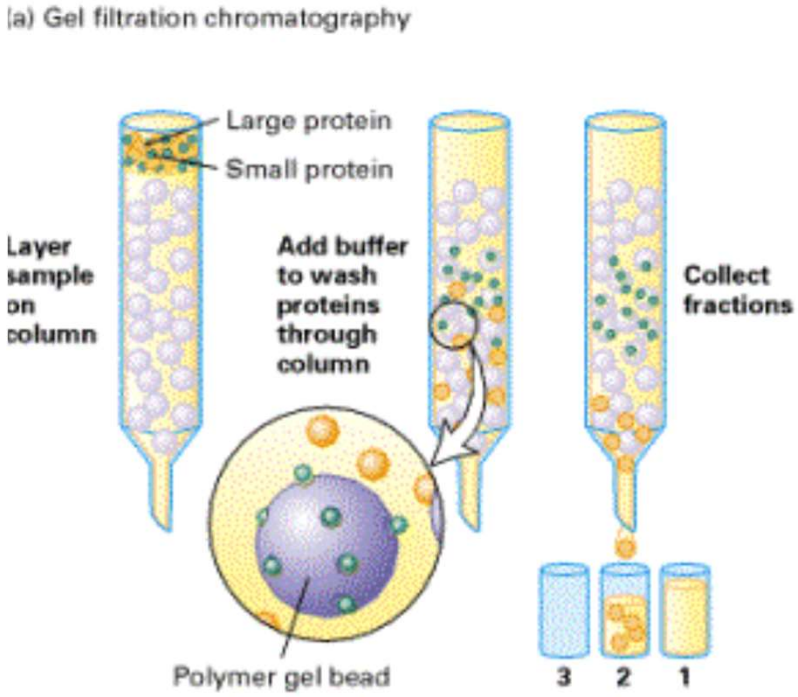


(B) Gel Filtration Chromatography:

Aim: Separate the protein bovine serum albumin (BSA, Mol. Wt. = 66,430 Da) from NADH (Mol. Wt. = 763 Da) using gel filtration chromatography performed with a pre-packed Sephadex dextran gel column.

Method:

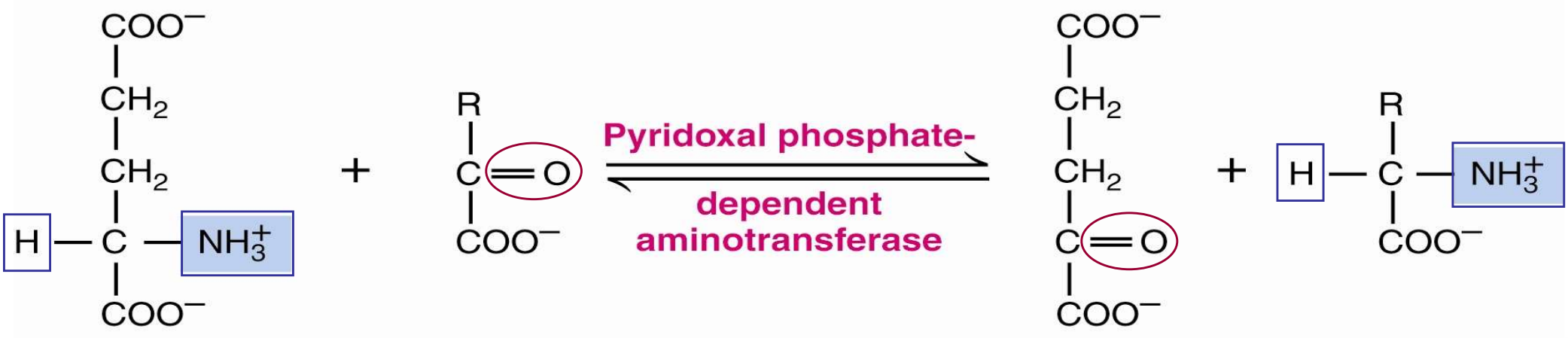
1. Setup column by washing 3 times with buffer.
2. Collect 6 drops of buffer in a weighing dish for weighing.
3. Add NADH-BSA mixture to the column, let it run until the liquid is just absorbed into the top of gel. Then, add the buffer to the top of the column.
4. Label 18 cuvettes from 1-18 and collect 6 drops of the NADH-BSA mixture from the column into each cuvette, in an order.
5. Measure the absorbance at 340 nm and 280 nm, using phosphate buffer as blank and enter into “Table 1”
6. Measure the void volume from the graph.



Prac 4 Excel worksheet C. Table 1

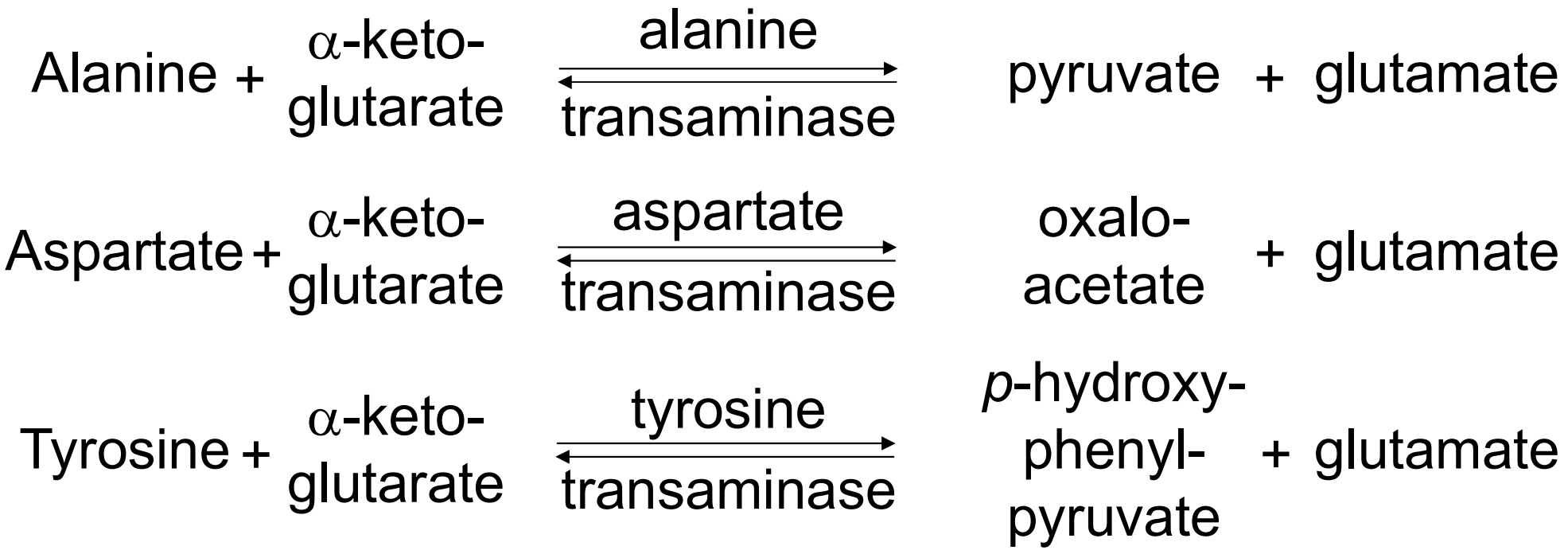
1. Label the graph.
2. Note if NADH and BSA get separated.
3. Report the void volume of the column.

(C) Transamination



- Transfer of α-amino group from specific amino acids to a limited number of α-keto acid acceptors
  - Pyruvate <-> alanine
  - Oxaloacetate <-> aspartate
  - α-keto-glutarate <-> glutamate
- Catalyzed by enzymes called “aminotransferases.”
- **Coenzyme**: pyridoxal phosphate (**PLP** - vitamin B_6).

(C) Transamination examples



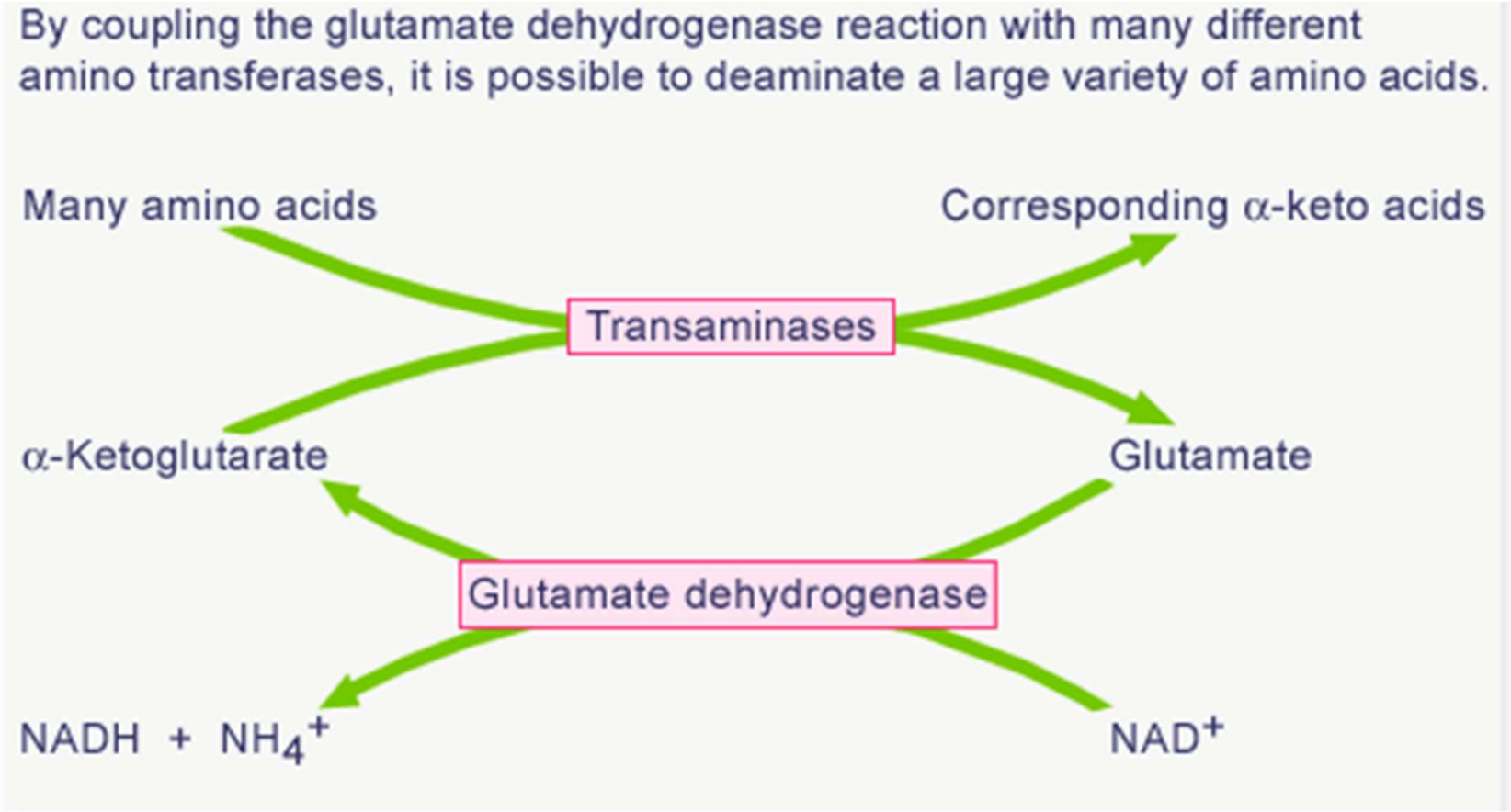
- Transamination reactions tend to channel amino groups to generate glutamate
- Transaminase reactions are reversible
- Important for amino acid metabolism.
- Important for exam

Role of Transamination

(i) Redistridution of α-amino groups to balance AA pool

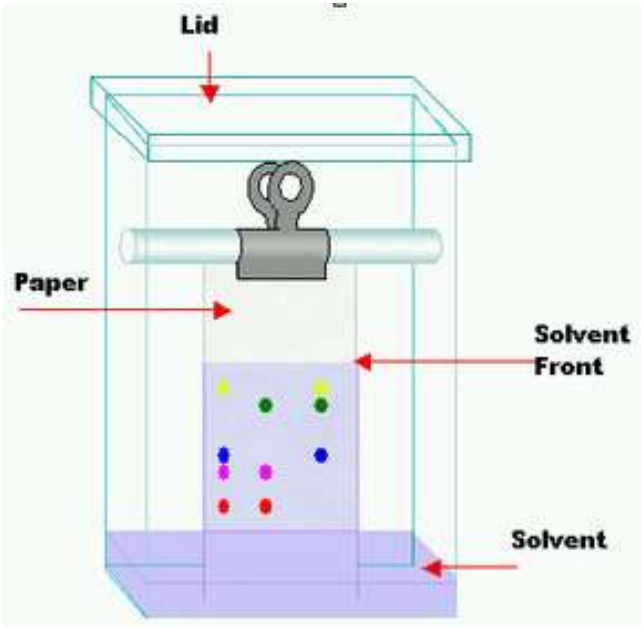
- Dietary proteins provide a mixture of AA whose proportions differ from AA pool required by body
- To correct imbalance

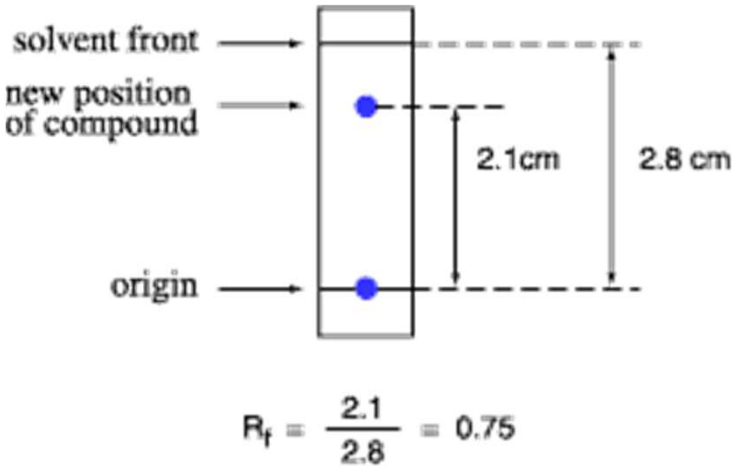
(ii) AA synthesis / degradation performed in conjunction with **glutamate dehydrogenase (GDH)**



Paper Chromatography

- Cation/anion exchange chromatography based on charge interactions
- Gel chromatography based on size
- **Paper chromatography** is based on **solubilities**





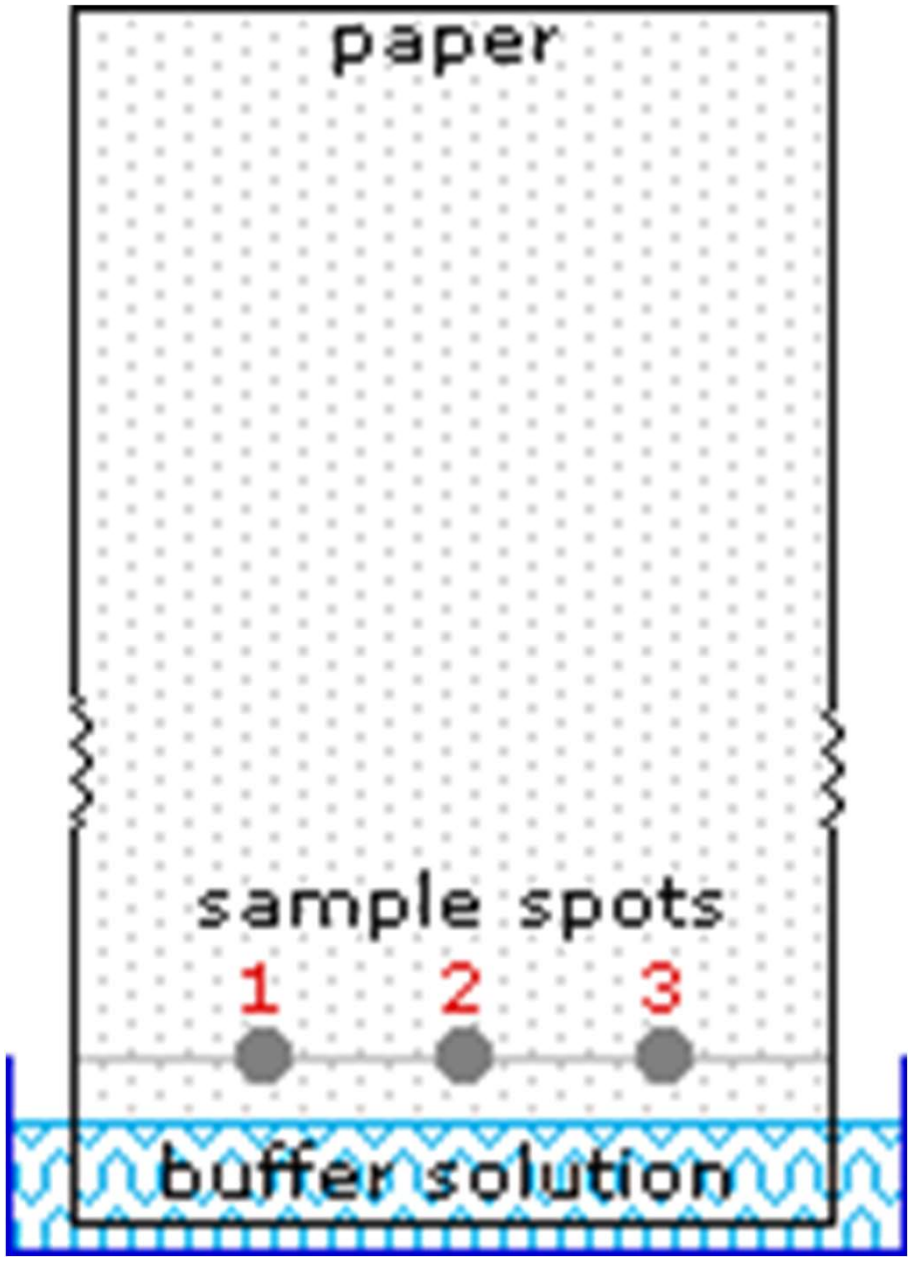
R_f value = Distance travelled by compound / Distance travelled by solvent

Solvent is drawn up the paper by capillary attraction

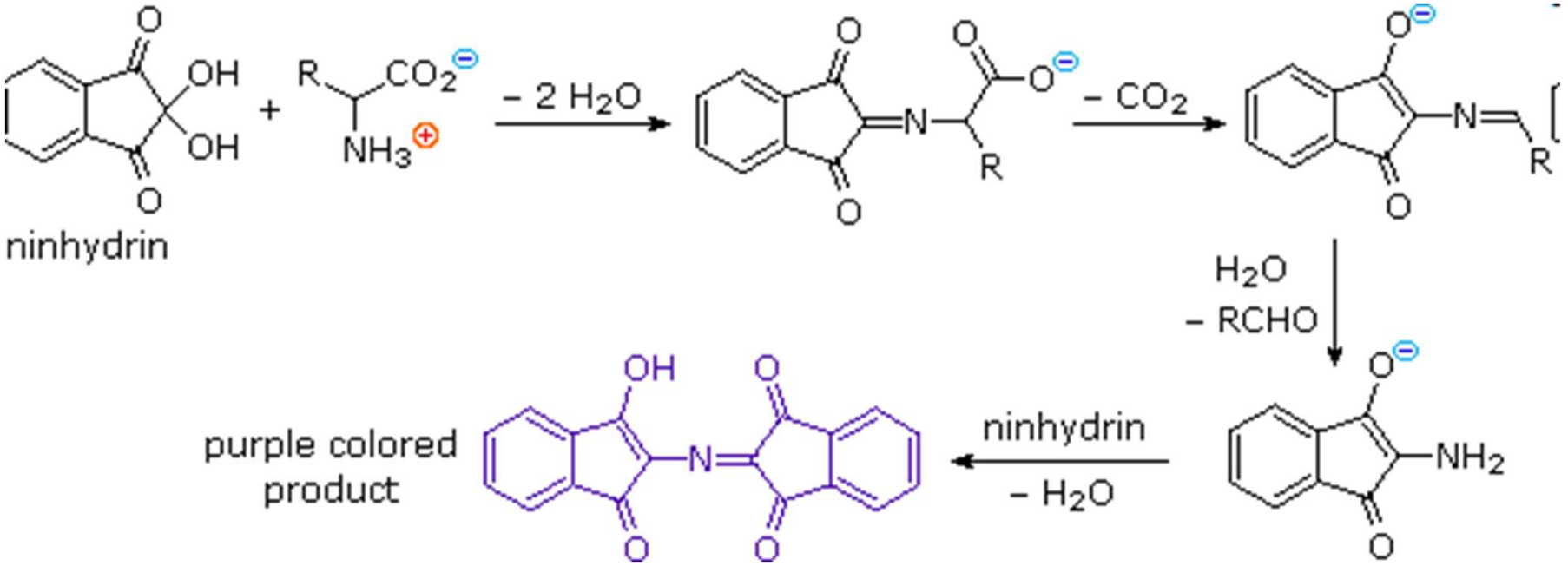
There is a tug-of-war between the compound sticking to the paper and being dissolved by the solvent, hence **different compounds travel at different rates** as the solvent is drawn up the paper by capillary attraction.

Paper chromatography - general procedure

- Spot the samples and reference compounds on the line
- Suspend the paper so that the line with the spots is a little above the buffer level
- When the solvent front almost reaches the top, remove from solution
- Dry and spray with reagent
- Mark the coloured spots and measure distances travelled by the solvent and each substance.
- Calculate R_f values



Ninhydrin reaction



- Ninhydrin requires **a primary or secondary amine** to produce usually a **purple** colour – this might fade with time.
- ***No colour with keto acids or aldehydes***.

(C) Transamination (done for you)

Students to do the calculations ONLY

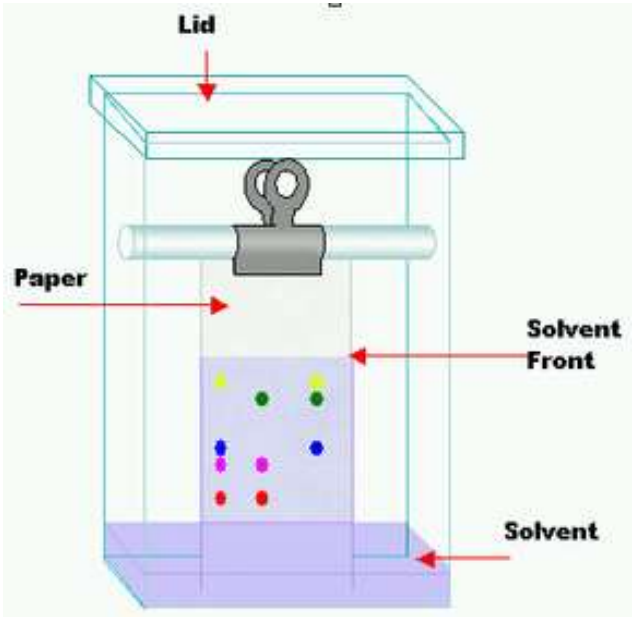
- Aim: Examine the **reversibility** of the reaction catalysed by the enzyme, **glutamic pyruvic transaminase (GPT)**

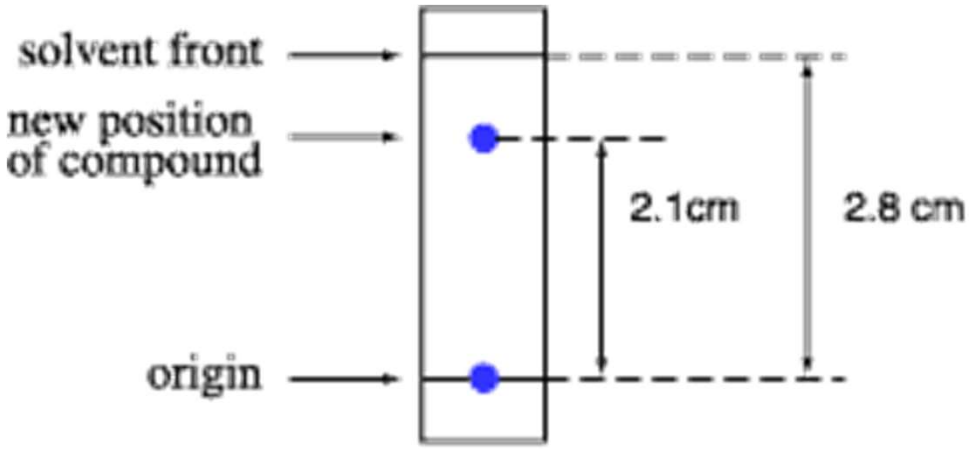
Note: this Prac takes **about 4 hours** to complete - so we have done it for you, so you can do only the data analysis.

1. Measure distances on the chromatogram provided to you.
2. Calculate R_f values for each spot and record them in **Table 3**.
3. Answer all questions

Results:
- **Fill Table 3**
- **Calculate R_f values.**

Answer questions.

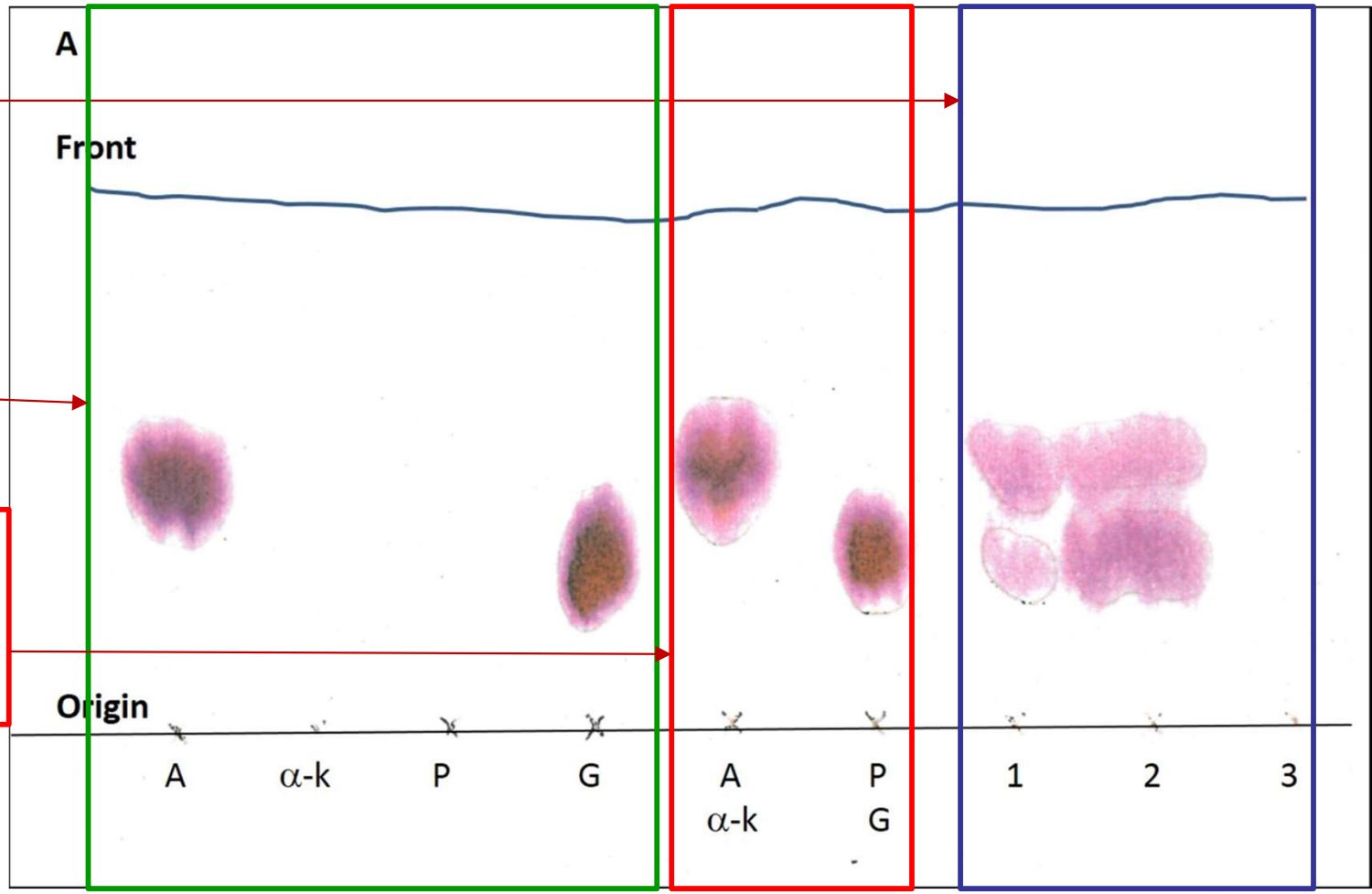




$$R_f = \frac{2.1}{2.8} = 0.75$$

Identifying molecular species in Reactions 1-3

- Compare R_f values for each spot in Reactions 1-3 with R_f values for:
  - pure substrate and product species
  - mixtures of pure substrates and products
  - Choose the **nearest R_f value** and write down the molecule name



A. Salting Out (Demo)

- Observe carefully the demonstration being carried out by your tutor.
- Record your observations.

A. What you need to do

- Go to iLearn and open the Prac 4 Data file (Excel).
- Go to the worksheet labelled: “A. Salting out (demo)”
- Record your answers to questions (a), (b) and (c).
- Save the Excel file.

Now you have completed Part A of the experiment.
Proceed to Part B.

B. Separate the protein Bovine Serum Albumin(BSA) from NADH using gel filtration chromatography (separation of nucleic acids/DNA from proteins)

Before you start the experiment

- View the Lab Safety video
  - ***Only the Column video will be shown in the lab briefing***
  - You can view the other videos before the prac or after the briefing.

Prac 4 relevant videos guides for setting up and using equipment:

- **Column video**
- Micropipette video
- Spectrophotometer video
- Waste disposal video

Please remember to complete your Pre-lab Quiz 4!

Lab Technique video

BMOL2201/BMOL6201
Biochemistry and Cell Biology

Using a **Sephadex column for gel filtration chromatography**

B. Procedure: Step 0

What to do - all students

1. You need **covered shoes**!

2. No **bags** on lab bench – place **in under-bench storage**

3. **Tie** up your **hair**, if applicable – **<u>tuck long hair into coat</u>**

4. **Button up** your **lab coat**

5. Put on **safety glasses**.

6. No food or drink please!

7. No laptops during experiment.

Need help?

- Lab Safety video

Procedure: Step 1: Getting started

Each team to collect one tray and fill it as follows:

Student 1 (gloves on)

A. Collect from the backbench and put into the tray:

1. a **cuvette holder**
2. 19 cuvettes
3. 5 mL Pipette (green ring)
4. **100-1000 µl pipette** (blue ring)
5. **Tips for these pipettes**
6. **A tube with 0.1M phosphate buffer pH7, containing 0.1M NaCl**

Student 2 (gloves on)

A. Collect from the backbench and put into the tray:

1. a box of **Kimwipes**
2. an **RO water bottle** (capped)
3. a **liquid waste container**
4. a **used tip container**
5. a **whiteboard marker**
6. a **Parafilm square container**

- Take the tray back to your desk
- The rest of the solutions are on your desk – mostly in the esky.

Procedure: Step 2 – Part B

Student 1 (no gloves)

1. Login in with your student ID
2. Download and open Prac4 excel file, **save on Desktop** and fill in your team details on the first worksheet.

Student 2 (gloves on)

1. Place the liquid waste beaker under the column. Open the column cap and **tip** the **buffer** carefully to fill the column.
2. Open the bottom cap to allow the buffer to drip out until the liquid level reaches the top of the matrix (column material). Put the bottom cap back.
3. Repeat **twice** more.
4. During the washing, **collect 6 drops** of buffer into **a pre-weighed weigh boat**.

Need help?

- Column video

Procedure: Step 3 – Part B

Student 1 (no gloves)

1. **Note the weight** of 6 drops of the buffer in the Excel file. This is also its volume (assuming density = 1 gm/ml)
2. **Complete** the volume column in **Table 1**, using the volume of 6 drops. **Save**.
3. Collect **19 cuvettes. Label one cuvette** with **"B", and number** the rest: **1** to **18**.

Student 2 (gloves on)

1. Using a micropipette, add **0.5 ml** of the **NADH-BSA mixture** to the top of the column.
2. Open the bottom cap to allow the mixture to drip out until the liquid level **just reaches** the top of the matrix (column material). Put the bottom cap back.
3. Top the column with buffer.

Need help?

- Spectrophoto-meter video
- Column video
- Micropipette video

Procedure: Step 4 – Part B

Student 2 (gloves on)

1. **Open** the bottom cap and **collect 6 drops** into each of the **cuvette 1-18, one by one.**
2. ***Replace the bottom and top caps of the column.***
3. To each of the above cuvette from Step 1., add **3 ml** of water using a micropipette, cover with a paraffin square and **invert 6 times** to mix.
4. Pipette out **3 ml** (3000 µl) **water** into the **'B'** cuvette and place in the slot marked '**B**' in the spectrophotometer chamber.
5. **Place** cuvettes **1-5** in **slots 1-5** in the spectrophotometer chamber.

Need help?

- Column video
- Micropipette video
- Spectrophoto-meter video

You have already seen the micropipette and spectrophotometer videos in Prac 2

Procedure: Step 5 – Part B

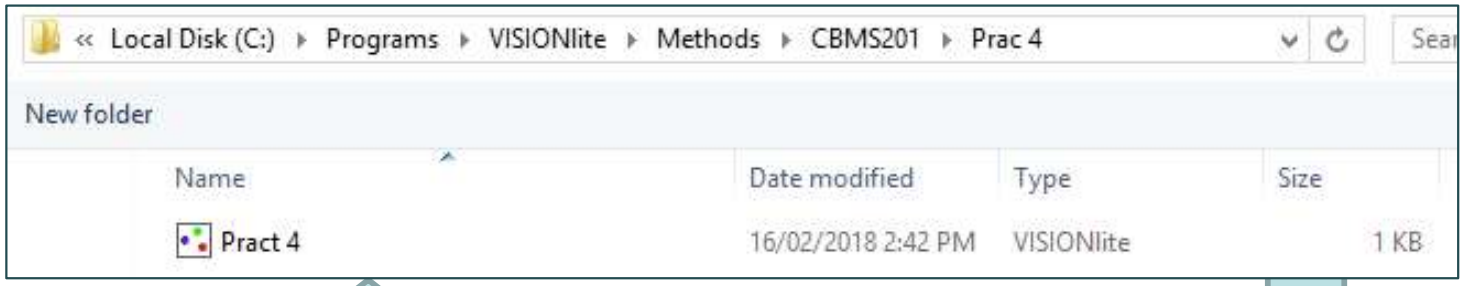
Two teams share the spectrophotometer by switching the connecting USB cord – pl. be patient. You may call your tutor to help.

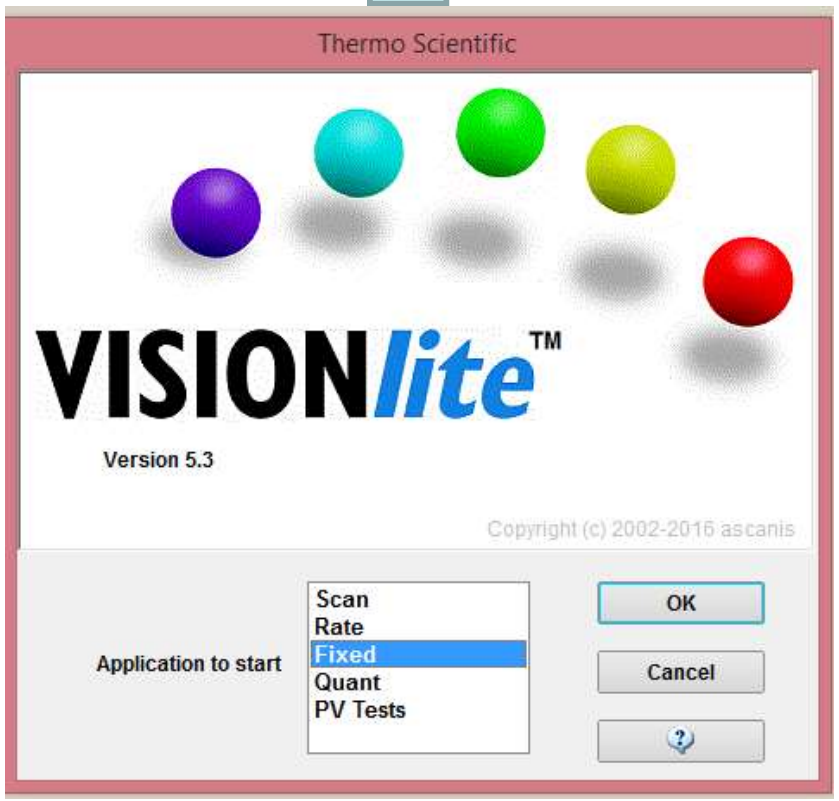
Student 1 (no gloves)

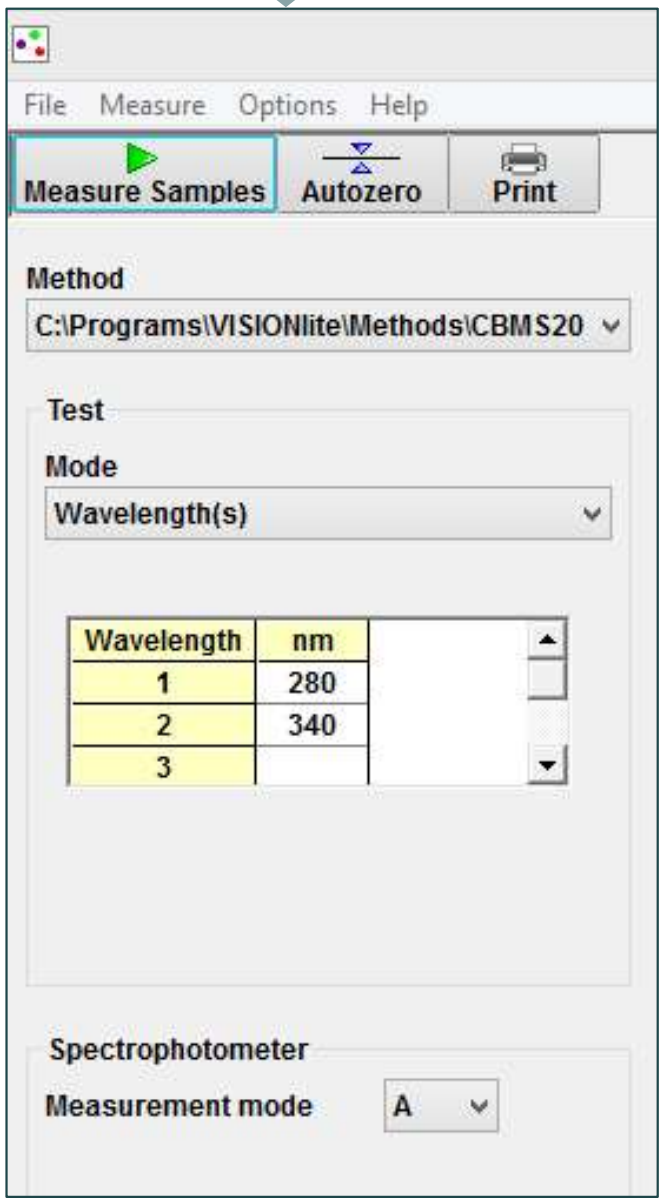
1. Double click on **Vision*Lite*** software on the Deskop and load the **Fixed** application from the Start menu (Check: the spectrophotometer screen should display **REMOTE MODE)**
2. Go to

   File→Open Method→CBMS 201→Prac4→PartB-Gel-filtration

   from the menu bar, to load parameters.
3. Click **Autozero** at the top of the screen. This correction will take almost 20 seconds
4. Click **Measure Sample** (at the top) and the sample information window will open up.
   a. Fill in your **name/initials** as the name of the **Result File** and click to select the **Measure Blank** option.
   b. Type in **Fraction 1** against location 1. Click **Fill Down**. This will populate all the locations with the numbers **2-5**.
5. Click **Measure** (at the top) and the **sample information** window will open up again.


« Local Disk (C:) ▸ Programs ▸ VISIONlite ▸ Methods ▸ CBMS201 ▸ Prac 4
New folder
Name
Date modified
Type
Size
Pract 4
16/02/2018 2:42 PM
VISIONlite
1 KB


Thermo Scientific
VISIONlite™
Version 5.3
Copyright (c) 2002-2016 ascanis
Application to start
Scan
Rate
Fixed
Quant
PV Tests
OK
Cancel


File
Measure
Options
Help
Measure Samples
Autozero
Print
Method
C:\Programs\VISIONlite\Methods\CBMS20
Test
Mode
Wavelength(s)
Wavelength
nm
1
280
2
340
3
Spectrophotometer
Measurement mode
A

Procedure: Step 6 – Part B

Student 2 (gloves on)

1. **Remove** the cuvettes from **slots 1 to 5 only - leave the blank in slot B.** Return each cuvette to the cuvette stand.
2. **Place** cuvettes **6-10** in **slots 1-5** in the spectrophotometer chamber.

Student 1 (no gloves)

1. In Vision*Lite*'s **sample information** window, type in **Fraction 6** in **Location 1** and click **Fill Down**, to populate all the locations up to 5.
2. Click **Measure**. When the measurement is complete, the **sample information** window will pop up again.

Student 2 (gloves on)

3. **After 2. above**, **remove** the cuvettes from **slots 1 to 5 only - leave the blank in slot B.** Return each cuvette to cuvette stand.

Procedure: Step 7 – Part B

Student 2 (gloves on)

1. **Place** cuvettes **11-15** in **slots 1-5** in the spectrophotometer chamber.

Student 1 (no gloves)

1. In Vision*Lite*'s **sample information** window, type in **Fraction 11** in **Location 1** and click **Fill Down**, to populate all the locations up to 5.
2. Click **Measure**. When the measurement is complete, the **sample information** window will pop up again.

Student 2 (gloves on)

2. **After 2. above**, **remove the cuvettes from slots 1-5, leaving the blank in slot B.** Return each cuvette to cuvette stand.

Procedure: Step 8 – Part B

Student 2 (gloves on)

1. **Place** cuvette **16-18** in **slots 1-3** in the spectrophotometer chamber.

Student 1 (no gloves)

1. In Vision*Lite*'s **sample information** window, type in **Fraction 16** in **Location 1** and click **Fill Down**, to populate all the locations up to 3.
2. Click **Measure**. When the measurement is complete, the **sample information** window will pop up again. Click **Close.**

Student 2 (gloves on)

2. **After 2. above**, **remove the cuvettes from all the slots including the Blank.** Return each cuvette to cuvette stand.

Procedure: Step 9 – Part B

Student 1 (no gloves)

1. From the Vision*Lite* window, go to **File→Export Results** and save results as **partB.csv** file on the desktop first and then copy it to your USB.

Student 2 (gloves on)

1. **<u>After the graphs are checked by the tutor</u>**, tip the solution from all the cuvettes and the liquid waste beaker, into the **Liquid Waste container**. *You may be required to collect additional samples.*
2. If all is well, place all the used cuvettes in the appropriate disposal trays.

- *Clean up your workspace*
  - *Check the spectrophotometer for that last cuvette left behind.*
  - ***<u>You may be marked 0 for participation and your work not graded if you do not complete this essential step.</u>***
  - *Get your tutor to check your place after returning your trays – thanks.*

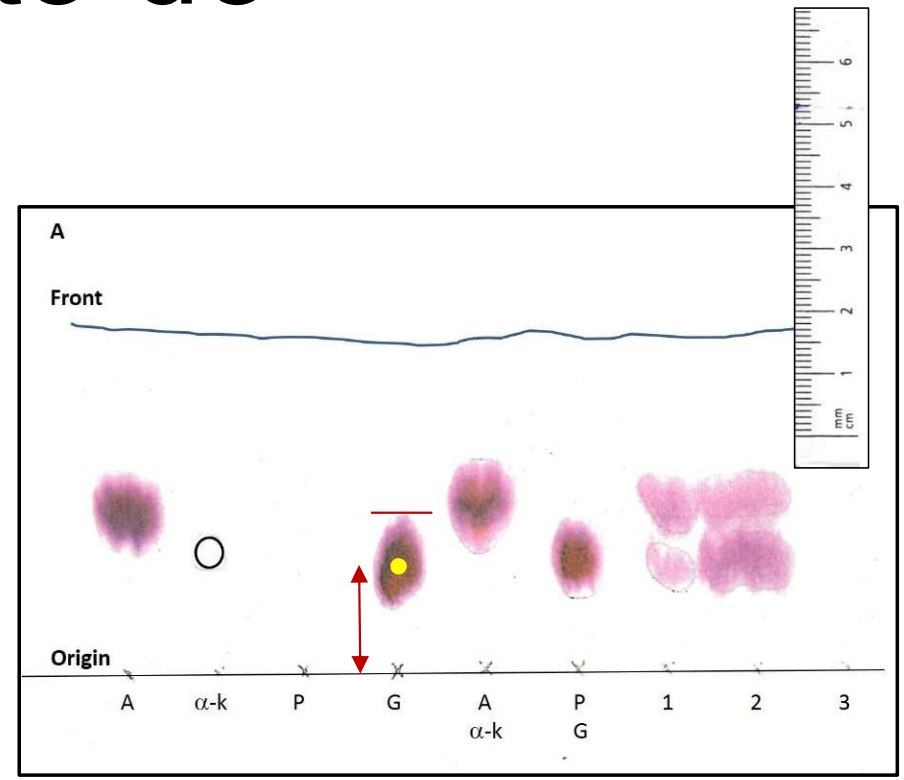
B. What you need to do

- In the Prac 4 Excel file, go to the worksheet labelled **'B. Gel Filtration Chromatography'**.
- Calculate the volume of **6 drops of buffer** eluted from the column. Use this value to calculate the "**Total volume eluted from column**" in **Table 1,** for cuvettes 1-18. Save.
- Download **partB.csv** file from iLearn. Double click on the **partB.csv** file, to open it with the Excel program.
- Select and copy the Absorbance values from tubes 1 to 18 in the column labelled "280nm" and "340nm"
- Paste the copied values into the columns labelled "Absorbance at 280nm" and "Absorbance at 340nm" in Table 1.
- **The graph should update itself.**
- Enter the graph title and the axes labels and save.
- Note the **Void volume (see Slide 16) from the graph.**
- Save the Excel file and go to Part C.

C. Investigate the **reversibility of an enzyme reaction,** catalysed by Glutamic Pyruvic Transaminase (GPT), using the separation method of **paper chromatography** *(application of separation technique to check out an enzyme reaction in metabolism).*

C. What you need to do

1. Go to the worksheet **C. Paper Chromatography** on your Prac 4 Excel file.
2. Using the ruler graphic provided, measure and record the distance from the origin (cross) to the centre of each coloured **Table 3**. *Hint: measure the top and bottom distances of each spot from the origin and take the average as "real" spots are fuzzy (see red lines and yellow dot).*



3. Measure and record the corresponding distance from the origin (cross) to the wavy line showing the solvent front, for each spot.
4. Using these two values, calculate the R_f values for each spot. By comparing with the R_f values of the standards, try to identify the amino acids present in each of the reactions **1-3**. Record this information in **Table 3**. Save.

C. Procedure: Step 10a – Packing up

Student 1 and Student 2 (gloves on)

1. **Wipe your bench clean with a Kimwipe.**
2. **Tip the solution** from all the cuvettes into the **liquid waste container.**
3. **Take the tray back to the back bench.**
4. **Tip the liquid waste into** the **liquid waste disposal container**.
5. Empty the **used tips** into their **respective trays/containers**.
6. **Wash** the following **using the water taps** and put them in the drying trays next to the sink:
   a. the liquid waste container and
   b. the used tip container.
7. Place **all used cuvettes** in the **collection tray**.

D. Procedure: Step 10b - Packing up

Each team to return the following to their respective collection locations

Student 1 (gloves on)

1. a **cuvette holder**
2. **5 mL pipette (green pipette)**
3. **100-1000 µl pipette (blue pipette)**
4. **Tips for these pipettes**
5. **The tube containing buffer**

Student 2 (gloves on)

1. an **RO water bottle** (capped)
2. a box of **Kimwipes**
3. a **whiteboard marker**
4. a **Parafilm square container**

- Leave the tray on the backbench.

Winding up

All team members

- *Clean up your workspace and return your tray.*
  - *Check the spectrophotometer for that last cuvette left behind.*
  - *Get your tutor to check your place after returning your trays – thanks*
- Complete all the calculations in the Data file and save the excel file on the desktop.
- Email the file to all team members, just in case.
- Complete the Prac submission individually as described in the next slide.
- You may use your laptops now, if there are no chemicals around.
- Submission due at the end of your practical session.
- *Remember to logout of your account on the computer!*
- *Take your USB drive with you!*

Submitting your data and Results

- Each student to login to iLearn and follow the instructions for completing the prac.
- **Upload your 'Prac 4 data file' individually to ilearn.**
  - ❖ Check your Prac 4 data file carefully to see if it is complete.
  - ❖ When you are 100% happy, upload your file to iLearn.
    - ➢ DO NOT EMAIL US YOUR DATA FILE AS ILEARN DOES NOT ALLOW US TO UPLOAD IT FOR YOU
    - ➢ IF YOU NEED TO RE-SUBMIT CONTACT GURPREET/ABIDALI/SHOBA, TO RESET YOUR SUBMISSION TO DRAFT
- You will then get access to Prac 4 Quiz
- Complete the Prac Quiz individually before you leave the lab!
- *We need your data file and completed Quiz for grading!*