Title: **Screening on the Microscale to Fight Emerging Pathogens**

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Abstract: As advanced technologies and automation become the norm in virtually every aspect of the scientific method, the need to introduce critical thinking skills at an early level becomes more important. This proposal will implement an analytical chemistry technique called crystallization by the hanging drop method to demonstrate the process of screening. The procedure can be introduced into the curriculum of secondary biology when discussing protein molecules and in physical science courses when discussing aqueous solutions. It is most suitable for a biotechnology course during the proteomics discussion. Students will learn to design a systematic method to set and maintain conditions for protein crystallization. Students will develop skill using microscale lab tools. Students will build a foundation of analytical experience and judgment that can be directly applied towards the urgent need to identify protein structures in various pathogenic agents.

Mission: The mission of this activity is to introduce and practice the concept of screening on the microscale by crystallizing lysozyme in the classroom with the hanging drop method. Students will design an experiment to empirically determine suitable conditions needed to grow lysozyme crystals. As a follow-on activity students may collect observations from a large number of trials and analyze the data using a computer and graphic representations to help identify optimum conditions for protein crystal growth.

Description: Screening on the Microscale to Fight Emerging Pathogens (SoMFEP) is a module designed to serve as an anchor activity around which interdisciplinary concepts in Biology, Chemistry, Biotechnology can be linked according to the individual needs of the classroom teacher. Enclosure (1) of the attached lesson plan contains a list of the relevant Florida Science Standards. Module One, the lecture, is suitable for any secondary Biology, Chemistry, or Biotechnology course. Module Two, the lab, is suitable for an advanced Biology or Chemistry course and any Biotechnology course.

The lab activity involves three stages (solution preparation, drop placement, and crystal formation) and can be accomplished in one 45 minute class period. It is intended to introduce the concept of systematically creating, maintaining, and monitoring conditions that will promote spontaneous formation of protein crystals under controlled conditions. Students may have previous experience with this process while making rock candy from a solution of table sugar and water. The hanging drop method employs the same principle on a much smaller scale. Crystals should form within two days and can remain stable for weeks if left undisturbed on the storage shelf. All reagents are water soluble and innocuous with disposal procedures described in the attached lesson plan.

Plan of Action

I. Background.

Screening is a significant and fundamental element of scientific inquiry. While this SoMFEP lab activity is primarily intended for chemistry students it can obviously be included in any of the advanced bioscience courses. Technologies and applications for screening may evolve over the time. However, scientists must keep in mind the indispensable need to systematically examine a wide range of conditions in order to determine suitability of a certain process. In this activity students will be presented with the problem of growing protein crystals under controlled laboratory conditions with the primary variables being concentration of the solution, pH of the solution, and temperature. For simplicity, students should focus only on concentration as the independent variable for each trial cell. Preliminary and follow-on activities might include students completing a series of concentration calculations and free response

questions to asses their understanding of the crystallization process. Additionally, this activity introduces the concept of vapor diffusion within a closed system which may be new to the students. Although it is essential to the success the lab, vapor diffusion would be less important for the discussion of Emerging Pathogens and more applicable for discussions in Chemistry and Physics.

II. Implementation.

This lab activity is intended to be scheduled as an anchor experiment in the spring semester of an advanced science course. An anchor experiment in this context is defined as a rigorous and relevant lab activity that will require students to practice advanced laboratory skills and apply previously acquired knowledge in order obtain a successful outcome. While the lab activity can be conducted within one class period, the pre-lab lectures, skill demonstration, practice, and post-lab assessments may extend beyond the scope a class period or two. Lab preparation guidelines, experimental procedures, and assessment recommendations are described in detail in the attached lesson plan along with a list of reagents, equipment, and instructional support materials.

As soon as funds are released and school year 2008-2009 begins, supplies for the lab activity will be ordered including one P20 micropipette. The single P20 will be used as a calibration standard for disposable plastic pipettes until the ICORE lab kit arrives. A concerted effort will be made to adapt procedures to utilize low cost and readily available lab equipment such as burettes, syringes, and disposable plastic pipettes. The approximate cost for consumables and lab equipment to develop this proposal is $400. Initial supplies have been donated by the Interdisciplinary Center for Ongoing Research and Education and the laboratory of Doctors Mavis and Robert McKenna. The balance of finding will be drawn from the Wellington High School internal account designated for the WHS Chemistry Honor Society.

The Module One slideshow has been previewed in classrooms across the Palm Beach District and the Module Two lab will be practiced in an Advanced Placement® Chemistry class in October 2008. Advanced Placement® Chemistry students have sufficient knowledge and experience to understand the concepts that will be reinforced during the crystallization procedure and offer meaningful feedback to guide refinements in the procedure. A full report of the outcomes will be finished by December 12th and submitted to the ICORE representatives in Gainesville for review and comment. The final presentation of outcomes will be reviewed and rehearsed prior to February 3, 2009.

III. Expected Outcomes.

After completing this activity students are expected to be capable of demonstrating competence in the following skills and concepts:

A. Calculating molar concentrations and volumes needed to make an range of solutions on the microscale (less than 1 mL).
B. Transfer of liquids using a micropipette.
C. The affect of concentrations of each component in a solution during the process of crystallization and precipitation.
D. The dynamics of solvent particle movement during vapor diffusion in a closed system.
E. The role of crystallization in preparation for X-ray crystallography.
F. The role of protein structural mapping in the fight against viruses.
G. The role of viruses in pathogenic diseases.

Expertise: Brian Nelson teaches Chemistry I Honors and Advanced Placement® Chemistry at Wellington High School. He has served for more than two years on the curriculum development team for chemistry in the Palm Beach District. In 2004 he worked as a summer intern at Scripps Florida Research Institute (Palm Beach Gardens) in the Medicinal Chemistry Lab under the direction of Dr. Bill Roush where he developed and implemented an interdisciplinary lesson module for on the synthesis of ester compounds. Brian earned a Bachelors of Science degree in Applied Science from the United States Coast Guard

Academy and is currently pursuing a distance learning degree for the Masters of Science in Science Education program at Florida State University.

Literature: *Chemistry: Matter and Its Changes*, Wiles and Sons, Fifth Edition, 2004

Budget and Budget Justification: The following list summarizes the supplies needed for the activity including the approximate costs and sources of supply:

					Unit cost	Total
Lysozyme protein reagent	1	1 g	BP535-1	Fisher	30.93	30.93
Immersion oil	2	4oz.	HR3-613	Hampton	15.00	30.00
Limbro® well plates	8	1 each	HR3-110	Hampton	6.00	48.00
Cover slips	1	box	HR3-229	Hampton	50.00	50.00
NaCl solution	1	500g	S671-500	Fisher	13.96	13.96
1-200 µL	1	box	02-681-151	Fisher	14.79	14.79
P20 micropipette	1	each		Fisher	250.00	250.00
				Grand total		$ 406.66

Screening on the Micro-scale to Fight Emerging Pathogens

Brian L. Nelson
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Lesson Plan: **Biotechnology (9-12)**
Biology (9-12)
Chemistry (9-12)

Module One: Slideshow and discussion on the role of viruses and protein crystallization in the study of emerging pathogens; suitable for all high school students. (30 min).

Module Two: Hands-on lab activity to set up a screening assay that will test conditions to form crystals of the enzyme lysozyme; suitable for advanced biology and chemistry students, and all biotechnology students. (30 min. for prep, 45 min. in lab with students).

Objectives:

Introduce protein crystallization as an important analytical step in the process of identifying protein structures.

Introduce the role of protein structures in viruses and the role of viruses in disease.

Introduce the principle of systemically screening through different conditions to determine empirically the most favorable conditions for growing crystals.

Practice solution preparations on the micro-volume scale.

Demonstrate a practical application of water vapor diffusion in a small closed system.

Florida Science Standards:

See Enclosure (1)

Prior knowledge and preconceptions:

Students should know the relationship between solute and solvent in a solution.

Students should appreciate that proteins are large macromolecules several orders of magnitude larger than molecules normally discussed in a first year general chemistry course. For example, the molecular mass of Lysozyme is approximately 14,000 amu.

Students should understand that a crystal is a repeating symmetrical pattern of ions, atoms, or molecules that can form when these particles are held together by intramolecular or intermolecular forces.

Students should appreciate that knowledge of protein structures can be used to explain the functions of the proteins and, further, that knowledge can be used to influence how viruses affect living organisms.

Module One: Why do we study protein crystallization?

Reading assignment:

"X-ray diffraction and Biochemistry", page 557, Brady and Senese (2004), *Chemistry: Matter and Changes*, 4th edition, John Wiley and Sons, Inc.

Proteins are the main building unit of all living creatures and essential components of all information and energy processing involved in life. Hence, understanding of genome-structure-function correlations for this group of natural compounds has emerged as a focus of intense recent investigations [1]. Detailed knowledge of the structure of the specific proteins that make up viruses can be used to prevent the spread of diseases in which a virus is the vector. The most widely used means for protein structure studies is still **diffraction of x-rays, electrons, or neutrons, by protein crystals**. To create images of atoms and molecules that are typically 1.5 –

2.0 Å apart these diffraction methods require single crystals as large as several tenths of a millimeter in all three dimensions. They should be mostly free of defects and structurally uniform. Thus, the preparation of diffraction-quality crystals is emerging as the bottleneck in the study of macromolecular structure [2, 3].

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1. Darby N.J.; Creighton, T.E. *Protein Structure*; Oxford Univ. Press: Oxford, 1993.
2. DeLucas, L.J ; Bugg, C.E. *Trends in Biotechnology* **1987**, *5*, 188.
3. Weber, P. in *Advances in protein chemistry, Vol. 41*, edited by Afinsen, C.B.; Richards, F.M.; Edsal, T.J.; Eisenberg, D.S.; Academic Press: New York, **1991**.

Presentation Materials:

The attached slide show gives some preliminary information and should be viewed prior to the lab activity.

Assessment:

See Enclosure (3) 10 question post lecture quiz.

Module Two: Screening for Crystallization Conditions

Pre lab preparation includes three steps:

1. Obtaining suitable well plates and covers (Limbro®).
2. Preparing Lysozyme and buffer solutions.
3. Obtaining a suitable micropipette drop delivery instrument. This procedure uses a model P20 micropette with user instructions found in Enclosure (2) [Adapted from Buffalo State University]

Lab Procedures:
[Adapted from Dr. Mavis McKenna and Dr. Robert McKenna, professors of Biochemistry & Molecular Biology, University of Florida, Gainesville, FL]

Introduction

This lab provides hands on experience with the hanging drop protein crystallization technique. The successful crystallization of any protein requires screening a large number of conditions (for example, concentrations of precipitant, salt, and protein as well as variations in pH, temperature and drop size) to empirically determine the best conditions for uniform crystal growth. In more advanced procedures the addition of detergents, organic solvents, and other minor components may also be used to slow down the rate of protein precipitation in an effort to produce large, perfect crystals. Finding suitable conditions for the crystallization of a newly isolated and characterized protein could take anywhere from a couple of weeks to several months to many years. The driving forces for crystallization are still very much unknown.

The optimum conditions for the enzyme lysozyme have been well characterized with respect to crystallization properties. You will use the hanging drop method which relies on vapor diffusion, in which a drop containing lysozyme/precipitant solution is allowed to equilibrate in a closed system containing a reservoir of precipitant.

With vapor diffusion, the sample is at 50% of the concentration of the precipitant compared to the reservoir solution, sometimes referred to as the mother solution, and is less than that required for protein crystallization. Vapor diffusion in each sealed well (i.e., closed system) results in the net transfer of water from the less concentrated, hanging protein solution in the drop to the more concentrated reservoir solution in the well. This diffusion will proceed spontaneously, until the precipitant concentration is the same in both solutions. Once the concentrations are equal this transfer of water ceases and the resultant protein solution stays at dynamic equilibrium. If the concentration is suitable, visible lysozyme crystals will form and can be examined under a microscope to determine uniform shape and relative quantity.

Materials Supplied

Stock solutions:	Equipment and other supplies:
4M NaCl	Limbro® Plate
0.5M Sodium Acetate , pH 4.2	Forceps
Distilled H_2O	22 mm Cover slips (slides)
Lysozyme at 50 mg/ml on ice	Pipette tips (sterile)
	Immersion oil and applicator
	P20 micropipette
	Two wood splints

Use five wells of the Limbro® well plate to screen the range of conditions over which lysozyme might crystallize, with NaCl as precipitant, at a slightly acidic pH. The precipitant (well) solutions should set up as indicated in Table 1. For each cell you will be expected to **calculate** the final concentrations of each component of the reservoir solutions during the post-lab to report your final answer. Then use the given the stock solutions, and distilled H_2O to make five solutions of the desired concentrations and final volume of 1.00 ml in the well. Alternatively, you may usse a micropipette or burette to carefully add the indicated volumes of each component in each well. At the end of this activity each student should have 5 wells, each with 1.00 ml of precipitant solution and a hanging drop on the cover slip on top of the well. Each lab group will share one well plate with all four students' samples. Leave it labeled and covered in the holding tray.

Note: We will microfuge the lysozyme to remove non-crystalline nucleations (impurities) before setting up the drops. Each drop will be a mixture of 5 l enzyme solution and 5 l of reservoir solution for a total of 10 l.

Table 1. Preparations for precipitate solution (mother solution) for each cell

Student #1	cell A1	cell 1B	cell 1C	cell 1D	cell 1E
NaCl	.1 ml	.2 ml	.3 ml	.4 ml	.5 ml
$NaC_2H_3O_2$	.1 ml	.1 ml	.1 ml	.1 ml	.1 ml
DI H_2O	.8 ml	.7 ml	.6 ml	.5 ml	.4 ml

Drop Placement

Step 1.
Apply a thin layer of oil around the rim of each well (reservoir) in the Limbro® plate where you intend to set-up a screening condition (columns A thru E, in rows 1 thru 4 for each student).

Step 2.
Use forceps or fingertips to pick up the cover slips (slides) and air blow them to remove dust if required. Lay them out on top of the two wood splints or other suitable surface so they can easily be picked up after the drop is placed.

Note: Don't touch with bare fingers – this will leave grease, oil and contaminants.

Step 3.
Pipette 5 µl of lysozyme stock solution onto the center of each cover slip and discard the pipette tip. Then, using a fresh tip, draw 5 µl of the precipitant reservoir (or "mother") solution from the first well (for example, **A1**) and add it to the first drop of stock lysozyme solution. Gently pipette up and down to homogenize the mixture.

Step 4.
Pick up the cover slip with forceps or fingers and carefully invert it, without disturbing the drop. Water's adhesion will cause the drop to stick to the glass cover slip and "hang" on the bottom while inverted. Now place the slip on the rim to cover the first well. The oil will form an air tight seal between the slip and top of well.

Step 5.
Repeat steps 4 for wells **A2 to A5** until all students have filled five wells **A-E.**

Step 6.
Once all students in the group have successfully placed and sealed their five drops, place a tiny (the size of a BB) wad of clay in the corner of each well plate and gently place the cover over the plate. Write your group number and names on the cover and secure the cover with one piece of green tape.

Step 7.
Place the covered well plate on the holding tray and return all materials to the starting position. Dispose of waste solutions in the appropriate waste collection containers.

Crystals should form within three days and you'll be able to examine them during the next class period.

Screening on the Micro-scale to Fight Emerging Pathogens

Enclosure (1) Florida Science Standards

Nature of Science Body of Knowledge (Grades 9-12)

SC.912.N.3.5 Describe the function of models in science, and identify the wide range of models used in science.

Physical Science Body of Knowledge (Grades 9-12)

SC.912.P.8.2 Differentiate between physical/chemical properties and physical/chemical changes.

SC.912.P.8.6 Distinguish between bonding forces holding compounds together and other attractive forces, including hydrogen bonding and van der Waals forces.

Life Science Body of Knowledge (Grades 9-12)

SC.912.L.15.6 Discuss distinguish characteristics of the domains and kingroms of living organisms.

SC.912.L.16.10 Evaluate the impact of biotechnology on the individual, society, and the environment, including medical and ethical issues.

SC.912.P.17.20 Predict the impact of individuals on environmental systems and examine how human lifestyles affect sustainability.

SC.912.P.18.1 Describe the basic molecular structures and primary functions of the four major categories of biological macromolecules.

SC.912.P.18.4 Describe the structure of proteins and amino acids. Explain the functions of proteins in living organisms. Identify some reactions that amino acids undergo. Relate the structure and function of enzymes.

Enclosure (3) Module One: Why do we study protein crystallization?

Post-Quiz

True or False

_____ 1. Three major categories of pathogens include parasites, bacteria, and viruses.

_____ 2. The genetic material in a virus is enclosed in a durable coat made of carbohydrates.

_____ 3. Gene Therapy uses a virus to insert genes into a cell so that the cell can make proteins using newly introduced genes.

_____ 4. Proteins are macromolecules with a molecular mass about a thousand times larger than simple sugars like glucose.

_____ 5. A solution is a homogeneous mixture of two or more substances with a solute dissolved in a solvent.

_____ 6. The Hanging Drop method allows a crystal of protein to grow inside a drop of solution which is allowed to dry in the open atmosphere.

_____ 7. X-rays can be used to gather information about the structure of a protein which tells something about the function of the protein.

_____ 8. In order for a protein to be X-rayed, it must be large enough to be visible to the naked eye.

Free Response

9. List three factors that affect the rate of crystal growth.

a. _____

b. _____

c. _____

10. In your own words, describe what it means to screen variables and conditions while conduction an experiment?

_____

_____

_____

KEY KEY KEY KEY KEY KEY KEY KEY KEY KEY KEY

Post-Quiz

True or False

__T __ 1. Three major categories of pathogens include parasites, bacteria, and viruses.

__F__ 2. The genetic material in a virus is enclosed in a durable coat made of carbohydrates. (Protein)

__T__ 3. Gene Therapy uses a virus to insert genes into a cell so that the cell can make proteins using newly introduced genes.

__T__ 4. Proteins are macromolecules with a molecular mass about a thousand times larger than simple sugars like glucose.

__T__ 5. A solution is a homogeneous mixture of two or more substances with a solute dissolved in a solvent.

__F__ 6. The Hanging Drop method allows a crystal of protein to grow inside a drop of solution which is allowed to dry in the open atmosphere. (Closed system)

__T__ 7. X-rays can be used to gather information about the structure of a protein which tells something about the function of the protein.

__T__ 8. In order for a protein to be X-rayed, it must be large enough to be visible to the naked eye.

Free Response

11. List three factors that affect the rate of crystal growth.

d. ____pH_____Purity_____

e. ____Temperature_____External Forces_____

f. ____Concentration_____

12. In your own words, describe what it means to screen variables and conditions while conduction an experiment?

___Answers may vary and should include testing many sets of conditions to determine what combination of variables will produce the best results. This is an empirical process which means the results are obtained by experiment.

_____

Use of Micropipettes

Prior to lab you should understand:

- The function of micropipettes in the laboratory
- Basic parts of micropipette
- What volumes are measured with P20, P200 and P1000 micopipettors
- How to read the volume indicator on a P20, P200 and P1000
- How much each micropipettor costs and how they are paid for

I. Objective:

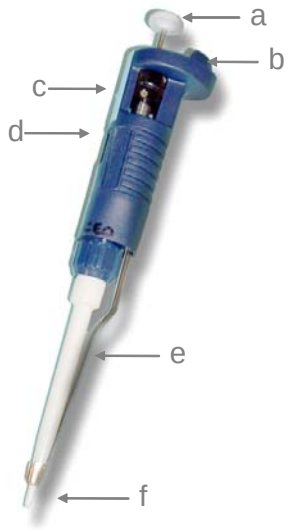
- **Learn how to use a micropipettor**

II. Background:

Micropipettors are the standard laboratory equipment used to measure and transfer small volumes of liquids. You will use them throughout this semester and in advanced courses that you take in the future. It is essential that you master their use if you are to be successful in your experiments.

A. Parts of a micropipette

a. Plunger button
b. Tip ejector button
c. Volume adjustment dial
d. Digital volume indicator
e. Shaft
f. Attachment point for a disposable tip



B. Three sizes of micropipettes

The micropipettors in this laboratory come in three different sizes each of which measures a different range of volumes. The three sizes are P20, P200 and P1000. These sizes are noted on the top of the plunger button.

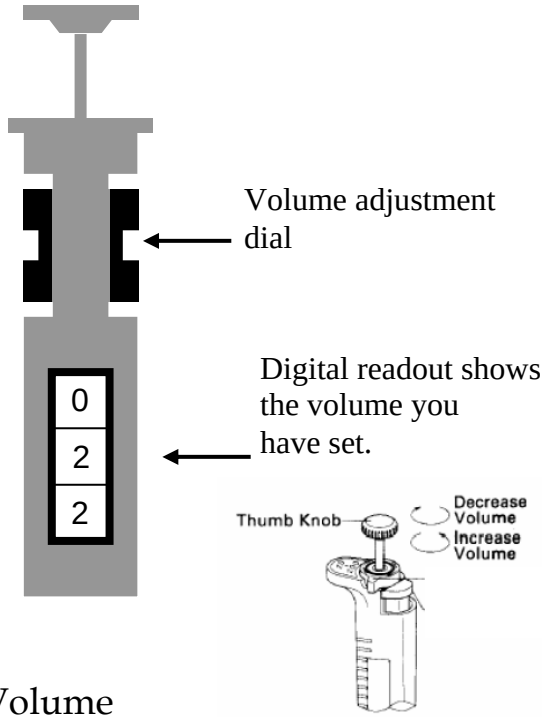
Size Micropipette	Range of volumes measured
P20	0.5-20µl
P200	20-200µl
P1000	100-1000µl

C. Adjusting Volume on micropipettes

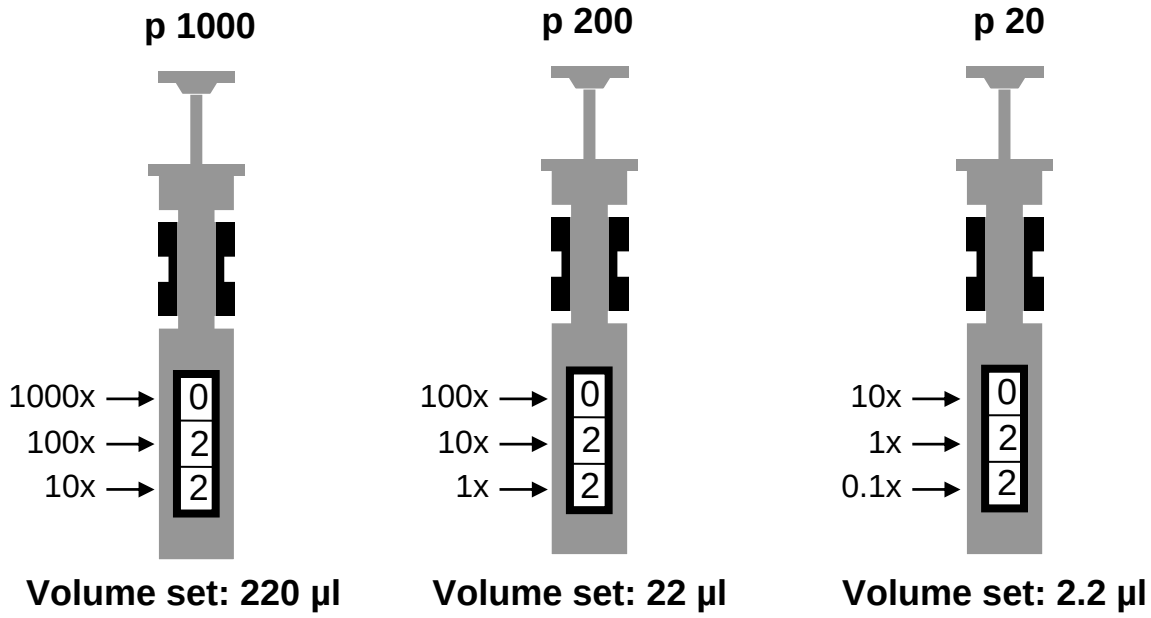
The black volume adjustment dial near the top of the micropipette allows you to adjust the volume that is measured. It can be dialed to the left or right to increase or decrease the volume.

The digital readout shows the volume that will be measured. As you turn the volume adjustment dial, the numbers in the digital readout will change.

On each of the three sizes of micropipettes (P20, P200, P1000) the digital readout has three numbers. These three numbers correspond to different volumes on the different size pipettes. See the figure below for instructions on interpreting digital readout.



Micropipettor: Reading the Volume



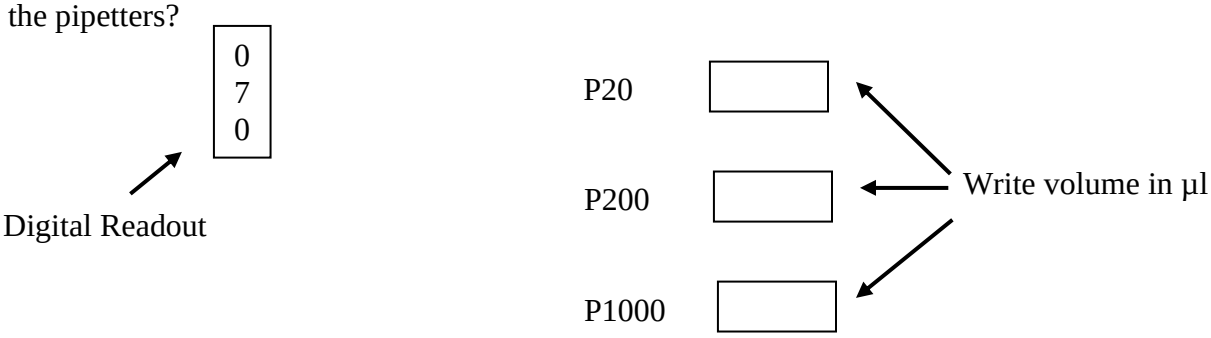
In a P100, the top number refers to 1000's of µl, the middle number refers to 100'sµl and the bottom number refers to 10's of µl's.

In a P200, the top number refers to 100's of µl, the middle number refers to 10's µl and the bottom number refers to µ'ls.

In a P20, the top number refers to 10's of µl, the middle number refers to µl's and the bottom number refers to $1/10^{ths}$ of µl.

Practice

In the boxes below, write how many µls the following digital readout correspond to in each of the pipetters?



Which micropipettor would be appropriate to measure 250µl? _____ Fill in the numbers that should appear in the digital display if that pipettor were to measure 250µl.

D. Pipette Tips

Liquids **<u>are never</u>** drawn directly into the shaft of the pipette. Instead, disposable plastic tips are are attached to the shaft. There are two sizes of tips. The larger blue tips are used for the P1000. The smaller clear tips are used for the P20 and P200.

The tips are racked in plastic boxes with covers. When you receive a box, it will be sterile. Please be careful when touching box or tips not to contaminate them. The box should be closed when not in use to prevent airborn contamination.

Inserting the Tip

1. Select the correct size tips.
2. Open the box without touching the tips with your hands.
3. Insert the micropipette shaft into the tip and press down firmly. This will attach the tip to the shaft.
4. Remove the micropipettor with the tip attached.
5. Close the box without touching the tips with your hands.

E. Punger Settings

The plunger will stop at two different positions when it is depressed. The first of these stopping points is the point of initial resistance and is the level of depression that will result in the desired volume of solution being transferred. The second stopping point is when the plunger is depressed beyond the initial resistance until it is in contact with the body of the pipettor. At this point, the plunger cannot be depressed further. This second stopping point is only used for the complete discharging of solutions from the plastic tip.

F. Measuring and transferring a volume of liquid

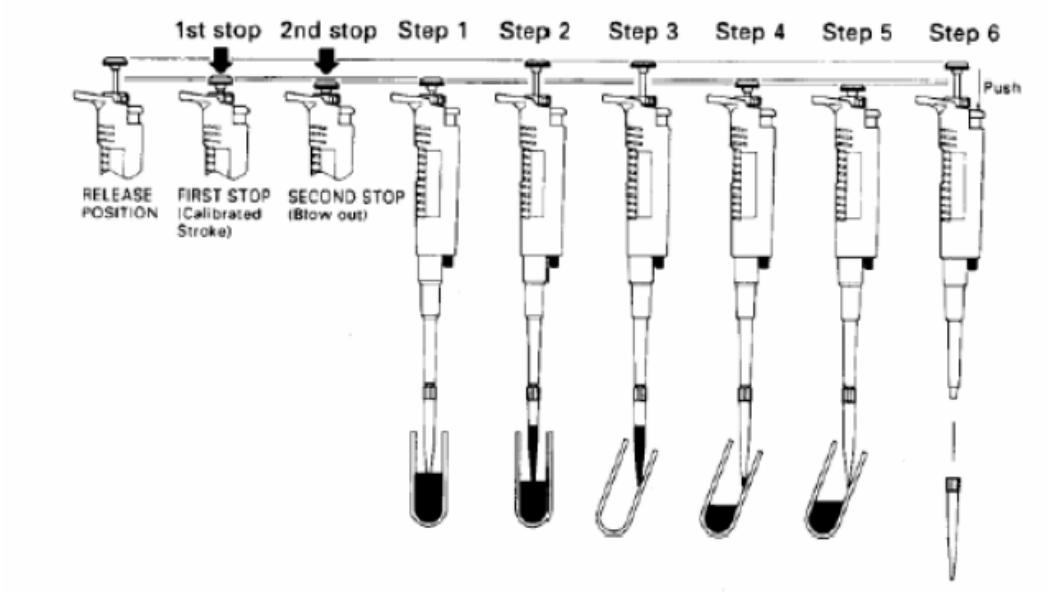
Before measuring and transferring liquid:

- Choose the appropriate size micropipettor
- Adjust to the correct volume
- Insert tip on the shaft.

Measuring and transferring liquid

The figure below shows the correct operation of the pipetteman. Important: note the first plunger stop is used in steps 1 through 4. The second plunger stop is only used in step 5.

- Depress the thumb knob to the first stop.
- Immerse the tip approximately 3 mm into the sample solution (step 1).
- Slowly release the thumb knob to the initial position (step 2). Watch as the solution is drawn up slowly into the tip. Do not release the plunger too quickly. Rapid release might draw bubbles in the solution and might splash solution on the non-sterile shaft.
- Withdraw the tip from the sample solution. Place the tip against the side wall of the receiving container (step 3).
- Smoothly depress the thumb knob to the first stop (step 4), pause, then depress the knob to the second stop (step 5).
- Remove the tip from the receiving container, and return knob to the initial position. Do not let the knob snap back.
- Remove the disposable tip by firmly depressing the tip ejector knob (step 6).
- Add as new tip and continue.



F. Micropipette Rules

Each micropipette cost $200 and is paid for by your technology fee. To keep these pipettors functioning properly it is important that they be handled with care. Please follow these rules to keep from breaking the micropipetters

1. Never adjust the volume beyond the range of the micropipettor. No micropipette should be adjusted below zero µl. The P20 should never be adjusted above 20µ, the P200 over 200 ul and the P1000 over 1ml.
2. Never force the volume adjustor dial. If the knob becomes difficult to adjust it probably means that you are exceeding the limits for the pipette or the pipette is damaged. Please report the problem to the instructor or TA.
3. Do not drop pipetters.
4. Always use a smooth motion when using the pipetters. This will help give you accurate measurements and also prevent breakage of pipettes. There should be not "snapping" noises.
5. Always keep pipettes upright. Store the micropipettes on the mounted rack on your bench when not in use. Never lay a pipette on the benchtop.
6. Always choose to appropriate size pipette for the volume you are measuring.
7. Always dispose of tips in appropriate waste containing. Never leave tips in glassware.

III. <u>Materials</u>:

Each Station should have:
P20, P200, and P1000
Small and Large tips
Microcentrifuge tubes
Disposable Cuvettes
Calibrated Spectrophotometer
Bottle of blue dye
Beaker of water
Liquid waste beaker
Container for discarded pipette tips
Water rinse bottle
Kimwipes

Note: We will be using a spectrophotometer to measure the amount of blue dye in each sample. We will discuss the how spectrophotometers work in future laboratory exercises. In brief they measure how much light a sample absorbs. The spectrophotometer passes a beam of light through a special sample holder called a cuvette. It then measures how much of this light is absorbed by the sample. The absorbance of light is displayed on the spectrophotometer. The more dye in a sample, the greater the absorbance.

IV. Procedure:

A. Preparing a dilution of Blue dye

1. Choose appropriate micropipettor to measure 75µl.
2. Transfer 75 µl of blue dye to empty microfuge tube.
3. Use a P1000 to transfer 1ml of water to microfuge tube.
4. Mix tube vigorously

B. Spectrophotometer reading

1. Pour the blue dye you diluted in the microfuge tube into a cuvette
2. Insert cuvette into spectrophotometer
3. Observe absorbance reading from digital display on spectrophotometer
4. Record measurement in data sheet below

C. Comparison to instructor value

1. Obtain correct absorbance value for 75 µl dilution from instructor
2. Divide your value by the instructor's value to determine whether you were within 10% of the instructor's value. If you are outside the 10% range you are making an error in your pipetting.

D. Independent trials

1. Repeat 75µl dilution two more times as outline in boxes A-C.
2. Rinse and tap dry cuvette between each measurement.
3. Use a new microfuge tube for each dilution
4. Record values in data collection sheet.
5. Compare with instructors value for same dilution

Repeating with other dilutions

1. Repeat procedures as outlined in boxes A-D except as outlined below.
2. Prepare new dilutions using the following quantities of blue dye
    a. 30µl
    b. 15µl
    c. 5µl
3. Record your data on the table collection sheet attached.
4. Conduct three trials for each dilution

V. Short Report **Name: _____**

Due at end of class session

Data Collection Sheet

Dilutions	Trials	Student Absorbance	Instructor Absorbance	Ratio (S/I)
75µl	1			
	2			
	3			
30µl	1			
	2			
	3			
15µl	1			
	2			
	3			
5µl	1			
	2			
	3			

Screening on the Microscale to Fight Emerging Pathogens

ICORE Project

Pathogens

In very general terms, the fight against disease causing pathogens can be divided into three classifications -

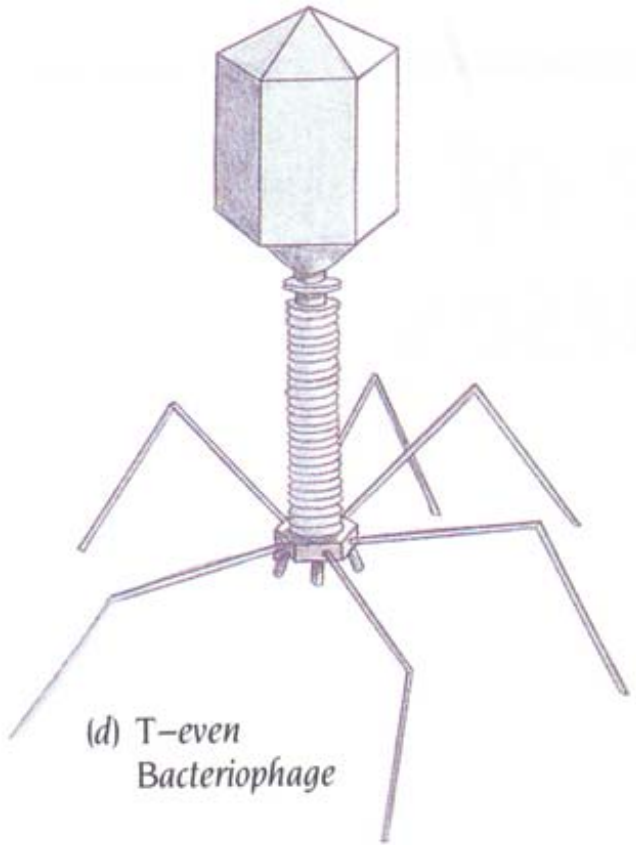
1. Parasites.
2. Bacteria.
3. Viruses.

Viruses

Recall what a virus is.

A virus is small mass of genetic material surrounded by a protein coat.

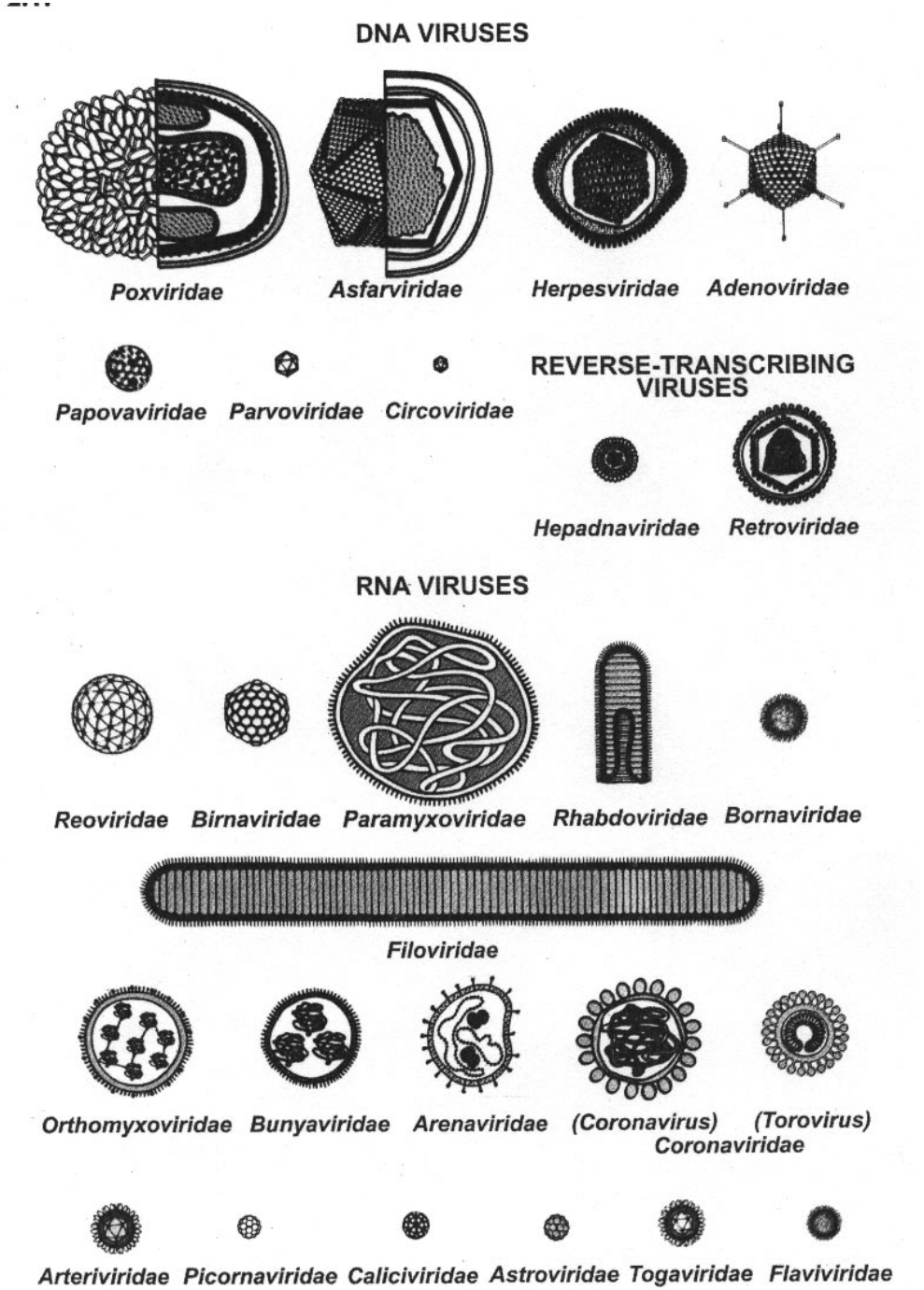
It reproduces in a host cell by hijacking the host's DNA replication process.

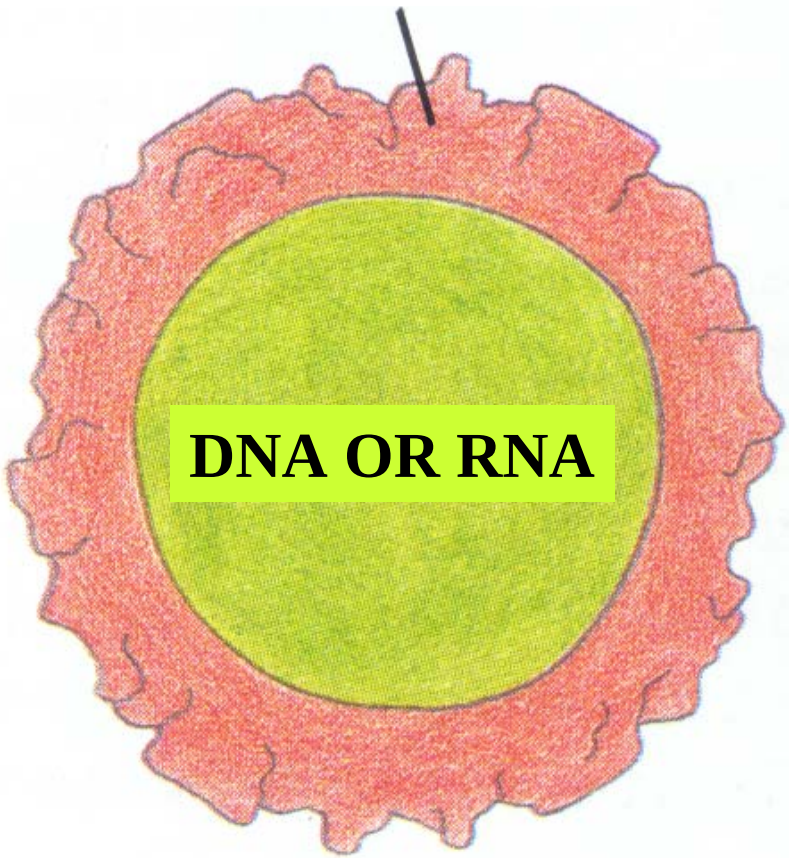


Viruses come in all shapes and sizes.

We can use advanced chemistry techniques to map the structure of the individual protein molecules that make up the coat.

Some of the proteins have unique functions we can identify and exploit.

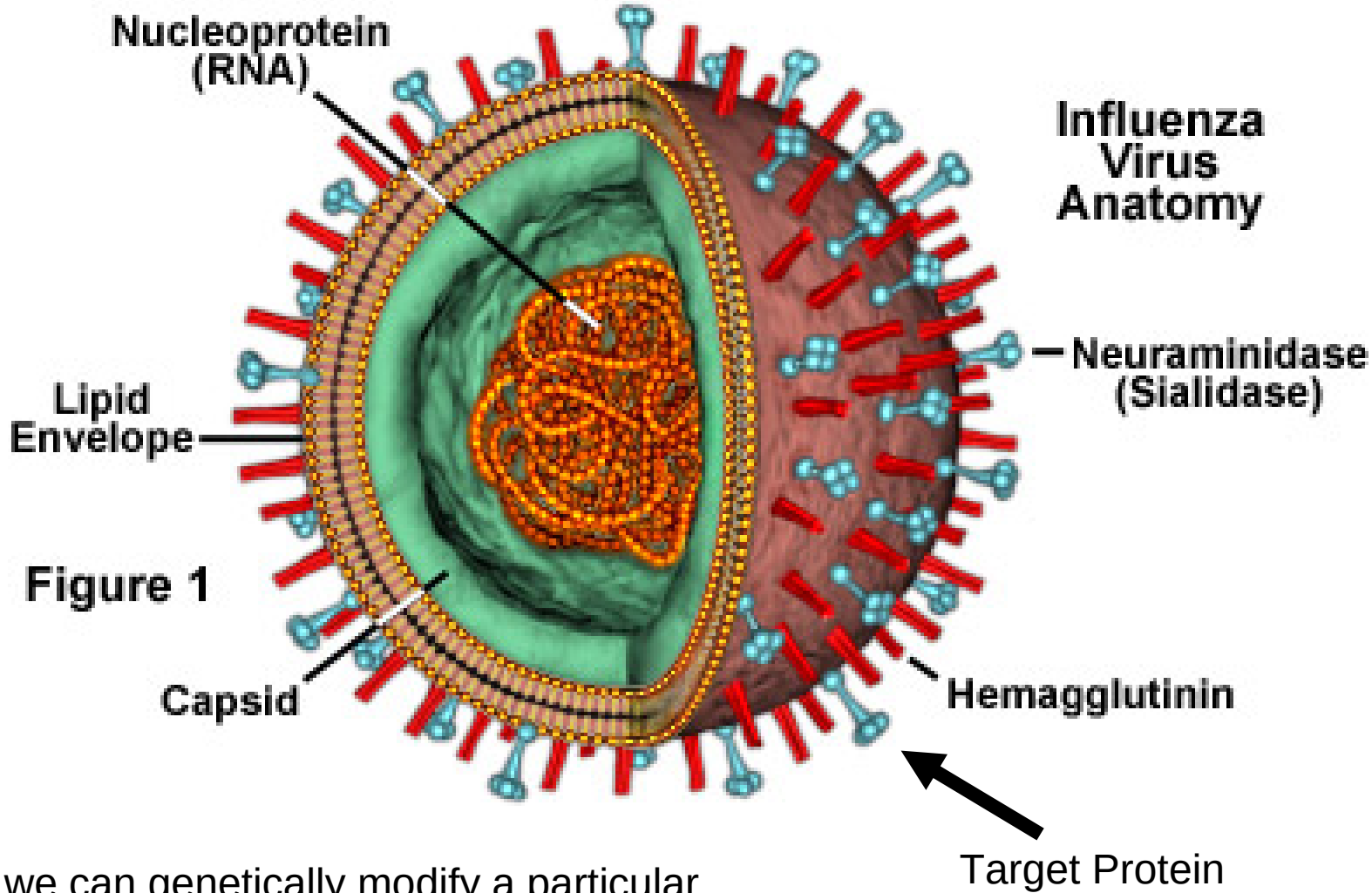




<u>Strategy</u> for the fight:

1. Identify and map the proteins in the coat and look for a weakness.

2. Disrupting their function can help to prevent the virus from attaching to the host cell's membrane.



If we can genetically modify a particular protein, then we might prevent the virus from penetrating the cell.

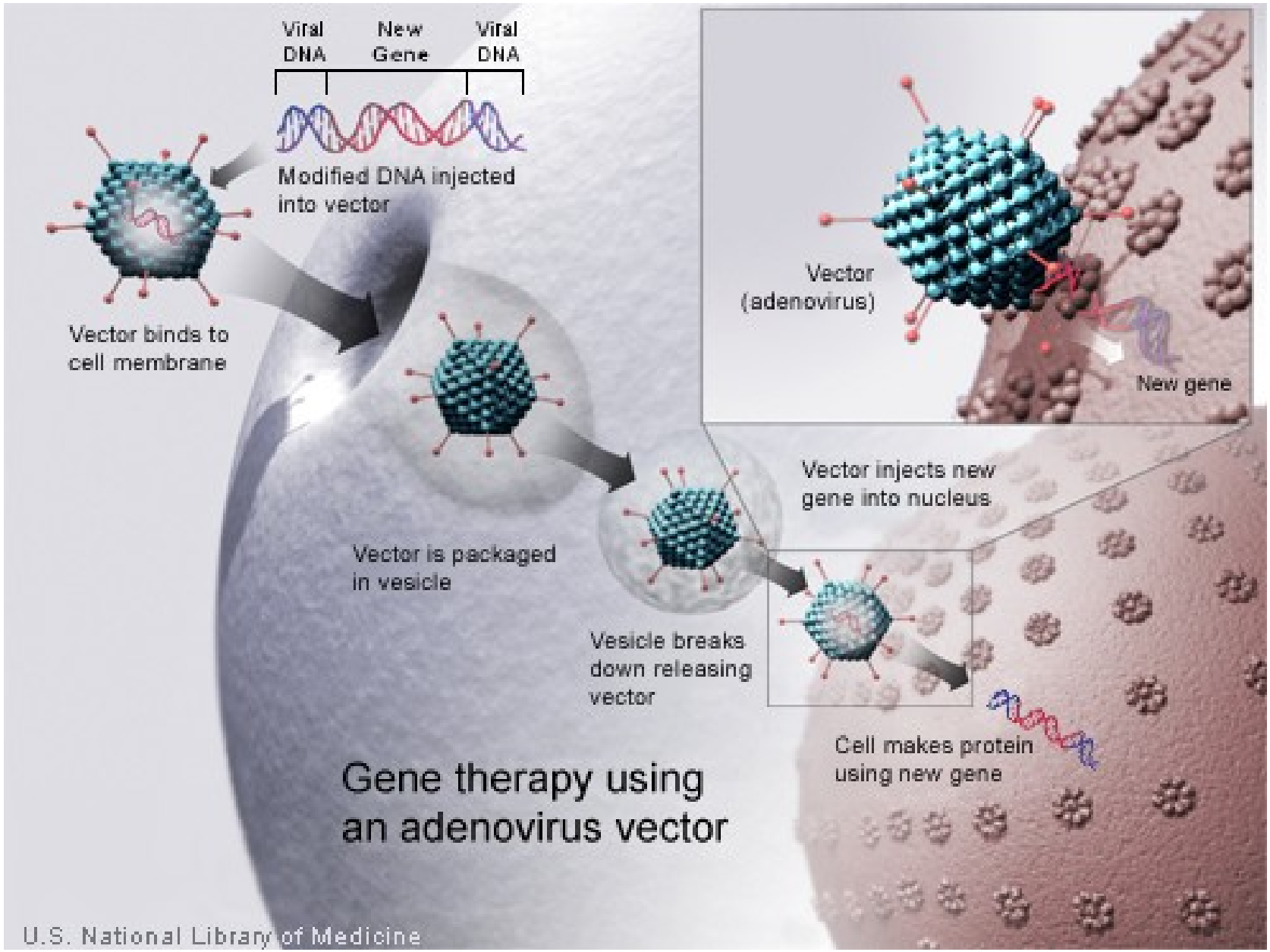
It Gets Better!

We can also turn the enemy to our side.

If we can gain enough information about the structure of the virus, *we can hijack it* and program the virus to deliver some new genetic material we want into the cell.

It's called "gene therapy."

Illustration of Gene Therapy, using a virus to insert genes.



Protein Crystallization

Our chemistry focus is on the **molecular** level.

Recall that proteins are macromolecules, very much smaller than the smallest cell, but still a thousand times larger than molecules we're used to seeing.

For example: H_2O, molecular mass = 18 amu

CH_4 , molecular mass = 16 amu

$C_6H_{12}O_6$, (glucose) molecular mass = 180 amu

Lysozyme, molecular mass is approximately 14,000 amu

Taking a Picture

In order to learn the structure of the protein we need to visualize the molecule with X-rays.

1. Isolate a sample of pure protein molecules.
2. Hold them still in a crystal.
3. X-ray them from different angles.
4. Analyze the X-rays to build a model of its structure.

Protein Crystallization

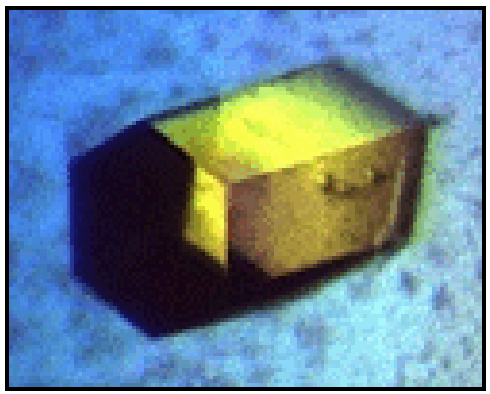
We will focus on making a crystal of protein molecules from a purified sample of an enzyme called lysozyme.

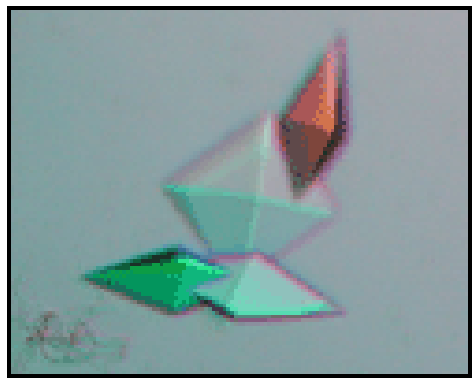
Once the crystal is made we can send it off for X-ray analysis.

There are thousands of isolated proteins just waiting to be crystallized and examined.

Crystallization by Vapor Diffusion

If we have a solution of protein (solute) and water (solvent), we can slowly dehydrate it until crystals start to form.





Crystallization is a Delicate Process

The rate and quality of crystal growth depends upon many factors such as…

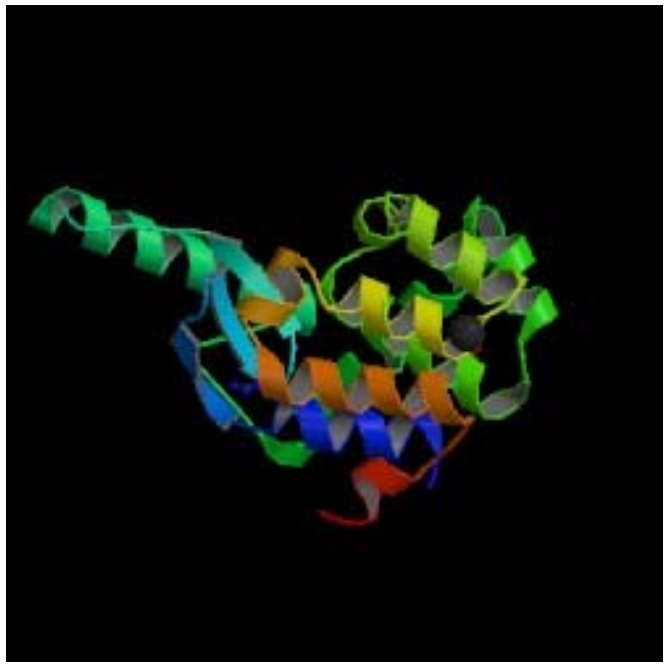
Concentration

Temperature

pH

Purity of the Sample

Gravity and External Forces



Lysozyme structure

Imagine building a house of cards

What factors would you want to control?

1.
2.
3.
4.
5.

Imagine building a house of cards

What factors would you want to control?

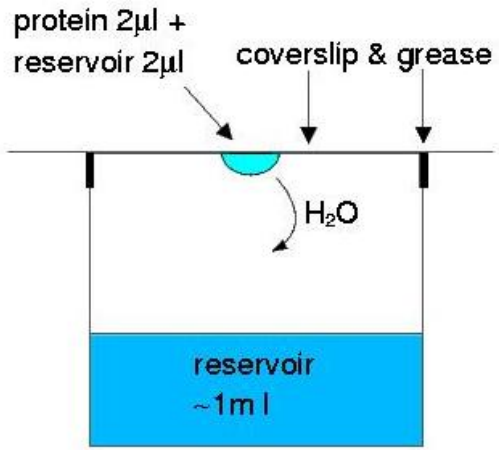
1. Maybe the type of cards?
2. Maybe the table surface?
3. Maybe the wind?
4. Maybe the dog running into it?
5. Maybe…

The Hanging Drop Method

We will try to make an isolated system where our sample can hang in an covered cell and slowly dry until crystals form.

We will try to control all factors and modify only the concentration of our samples.

Illustration of the **Hanging Drop Method**

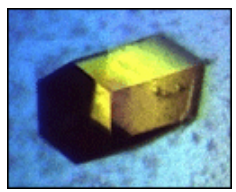


The well plate is sealed from the outside atmosphere with a cover slip and water diffuses from the drop into the reservoir.

Limbro® well plate

Recall that diffusion is the spontaneous movement of molecules from an area of high concentration to low concentration.

The protein molecules are much too large to vaporize so they will remain in the drop and form crystals!



Screening for the Best Conditions

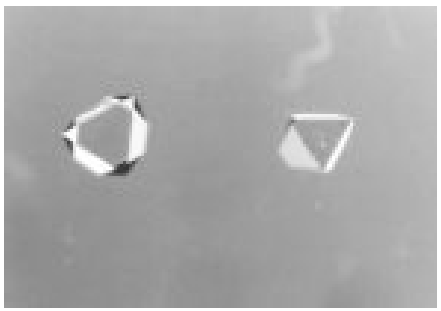
A fundamental principle of scientific inquiry is SCREENING many sets of conditions to determine which variables produce the most favorable results.

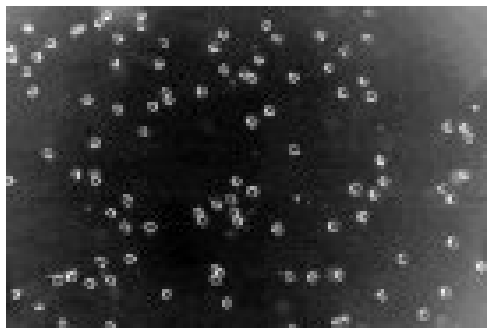
You will perform the same Hanging Drop Procedure under different conditions of concentration to determine what works best.

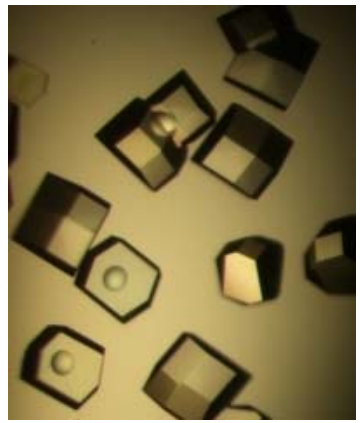
This is an ***empirical*** determination.

Seeing is Believing!

- This method should produce crystals of Lysozyme, within about three days, that are visible with the naked eye.







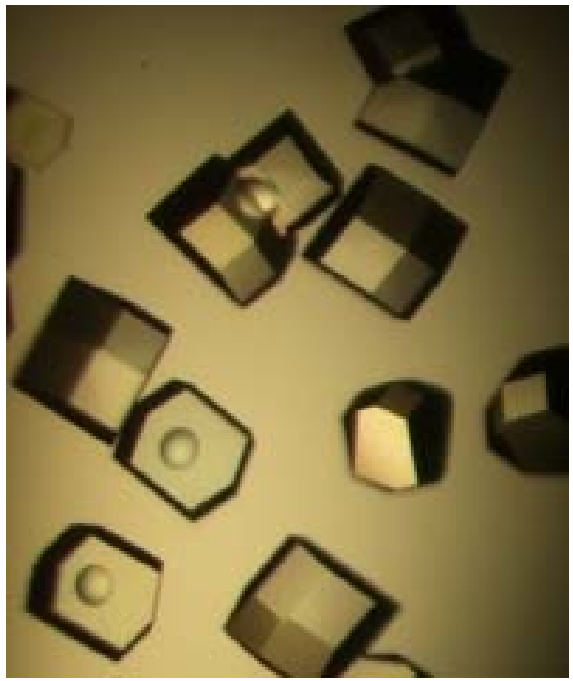
- Under a 3x binocular microscope or higher they appear as uniform 3D crystals with a definite shape and definite lattice angles.

Let’s give it a try!

Observe demonstrations of how to use micropipetts, the Limbro® well plates and cover slips, and drop placement.

Each student will make 5 cells to determine which conditions of concentration produce the best conditions for crystal growth.

Follow the Lab Procedures



Good Luck!