GENERAL PRACTICAL (I) INTRODUCTORY LECTURE

Steven Odongo

opodongo@yahoo.co.uk /sodongo@vub.ac.be

CMIM, Building E, Floor 8, Room E8.07

Vrije Universiteit Brussel

INTRODUCTION

Objectives:

- General practical (I) preparatory lecture.
- Good laboratory practice (GLP) (*see manual*)
- Learning basic laboratory techniques.

MAKING SOLUTION

(Reagents & Buffers)

a) BASIC CALCULATIONS

Calculate and re-check to ensure no mistake before starting buffer preparation

- *Molar solution*
- *Percent solution*
- *Concentrated stock solution*
- *Dilution of stock solution*

Molar solution

- Molarity = Weight of a solute (in grams) equals its FMW is dissolved in 1L of solvent

Calculation of Molarity (M)

- Know final volume (V), final concentration (M) & FMW of solute.

E.g. When asked prepare 1M NaCl (FMW=58.43) in 1L water (H_2O);

- Weigh 58.43g NaCl in a cylinder and fill with H2O to 1L (1000ml) mark

E.g. When asked to prepare 2M NaCl (FMW=58.43) in 1L H_2O;

- Weigh (2x58.43)g NaCl and fill with H_2O to 1L mark

➢ Alternatively use formula:

- FMW (g) x final volume (L) x final concentration (M) = grams required

E.g. To prepare a 500 ml (0.5 L) solution of 5M NaCl (FMW=58.43) in water;

- **58.43 x 0.5 x 5 = 146 g**

Percent solution

- Percent (per 100) solutions = based on 100ml or 100g of solution

Expression of concentration of solute as percentage (%)

- Percent weight by volume - % (w/v)
- Percent volume by volume - % (v/v)
- Percent weight by weight - % (w/w)

Percent weight by volume - % (w/v)

- *Grams of solute per 100 ml of solution*

E.g. 20% Glucose = 20g glucose dissolved in 80g (80ml) water.

Percent volume by volume - % (v/v)

- *ml of solute per 100 ml of solution.*

E.g. 1% ethanol solution = 1ml ethanol dissolved in 99ml water.

Percent weight by weight - % (w/w)

- *Grams of solute per 100g of solution.*

E.g. 10% sucrose solution = 10 g sucrose dissolved in 90 g (90ml) of water.

Concentrated stock solution

- Buffers may be prepared or provided as a *concentrated stock* (2x, 5X 10X etc…), to be diluted into a *working concentration* (usually 1x)

- ***E.g.*** *a 10x phosphate buffered saline (PBS)* ***per 1L of solution requires 10 times each reagent used for preparation of*** *1xPBS*.

If 1xPBS = 8g NaCl, 0.2g KCl, 1.3g $Na_2HPO_4.2H_2O$, 0.24g KH_2PO_4, per 1L solution.

Then for a 10xPBS, multiply each reagent by 10, top with solvent (H_2O) to 1L solution.

Dilution of concentrated stock solution

- Formula used commonly: $C_i \times V_i = C_f \times V_f$
- ❑ C_i = initial concentration, or concentration in stock solution.
- ❑ V_i = initial volume or amount of stock solution needed.
- ❑ C_f = final concentration or concentration in desired solution.
- ❑ V_f = final volume of desired solution.

E.g. 1) To prepare 100 ml of 0.05M solution from 1.5M NaOH

Ci = 1.5M, **Vi** = ?, **Cf** = 0.05M, **Vf** = 100 ml ➡ 0.05 x 100 = 1.5 x **Vi**

Vi = 3.33 ml of 1.5M NaOH (stock), add H_2O to 100 ml

2) To prepare 100 ml of 1XPBS buffer from a 10XPBS

b) PREPARING BUFFERS:

Gather all that you need

wighing boats

Spatulas

Beaker

Measuring cylinder

Erlenmeyer flask

Magnetic stir plate

Weighing balance

Magnetic stir rod

Weighing chemicals:

- Put on gloves
- Read instruction on the bottle (bse may require wearing mask)
- Turn on the balance & tare the weigh boat
- Weigh the chemical, pour in a beaker or flask (for toxic chemicals cover flask immediately)
- Close the lid of the stock jar & return it to the shelf immediately
- Weigh and clean the balance when finished

Mixing solute with solvent:

- Add 80% of the final volume of liquid solvent to the beaker followed by solid.
- Drop magnetic stir bar of an appropriate size
- Put beaker on the magnetic stir plate & slowly turn on the magnetic stir plate
- Stir until complete dissolution
- Pour solution into the graduated cylinder bring volume to 90%
- Pour solution back into the beaker for pH adjustment

C) pH measurements and adjustments

Calibration of pH meter

- Required: At least 2 pH standard solution (pH 4,7&10), 3 or 4 50ml beakers, wiping paper
- Rinse electrode and gently dry on wiping paper
- Pour ~1.5 inches of standard
- Press "standardize" and wait until pH stabilizes to standard's (e.g pH 4)
- Press "standby", rinse electrode and dry
- Repeat standardization for pH of 10 if not yet done.
- Rinse electrode, put back in storage buffer
- Read the pH of standardization buffers

Determining pH

Requirements:

- Magnetic stir bars and plates,
- (12.1 & 1)M HCl,
- (5 or 10)M NaOH & 0.1M NaOH.

Procedure:

- Stir the the solution on stir plate. Stir bar should rotate very slowly to avoid damaging electrode.
- Raise electrode out of soak beaker and rinse, then immerse into the solution which the pH is to be measured
- Change the function switch from standby to pH
- Wait for the pH to stabilise and adjust by adding NaOH or HCl

- Add a drop of (NaOH or HCl) at a time, stir, then measure
- If the pH is off by one unit, use more conc acid (12.1M HCl) or base (5 or 10M NaOH).
- Do not use the same dropper for adding acid and base
- Always will need more drops near pH at which the solution is buffered.

- Transfer the solution into a measuring cylinder, bring the volume to 100%
- Pour the solution into glass or plastic bottle with cap
- **Label bottle**:- date, content, concentration, pH and your initials.
- Put a piece of autoclave tape. Dark strips appear after autoclaving.
- If not autoclaving immediately keep at 4° C

D) Sterilizing solution:

1) Autoclave:

- Most buffers
- Undefined bacterial and yeast media

But don't autoclave:

- Corrosives (acids, bases & phenols), volatile solvents (-OHs, Chloroform), or radioactive materials, liquids containing beach (sodium hypochlorite), formalin, or glutaraldehyde, heat labile ingredients (serum, antibiotics, proteins & vitamin), mammalian, plant, and insect media, HEPES containing solution, DTT or βME solutions, buffers with detergent such as 10%SDS.

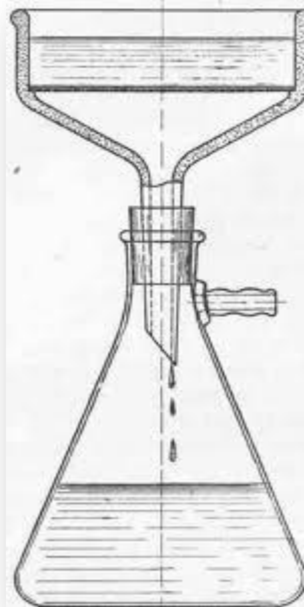
2) Filtration

- Recommended for heat labile or volatile or solution less than 20ml.

- Viscous solution is pre-filtered through 0.4µm pore. a 0.2 µm (most media and buffers) or 0.1µm (tissue culture media)

- **NB:** Virus will not be filtered out

Syringe filters



Vacuum filters

e) Storing buffers and solutions

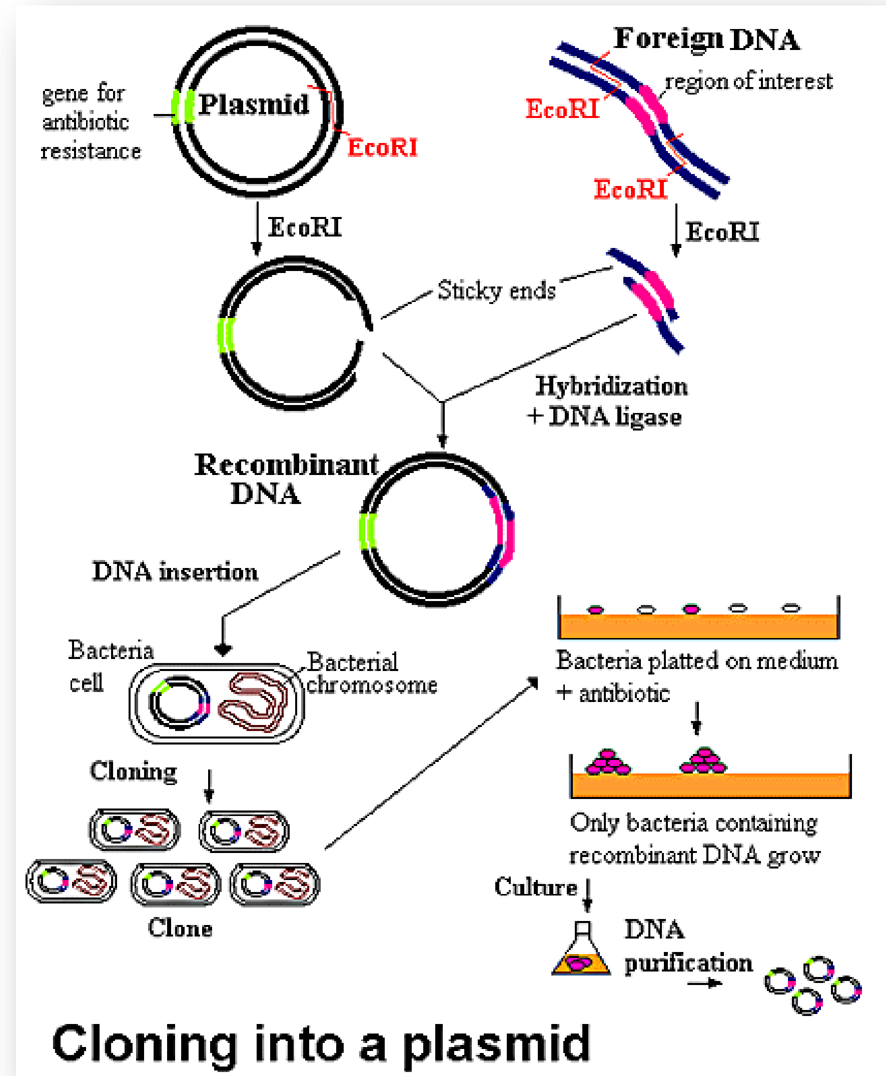
- Store media at 4 ° C
- Store buffer at room temp or 4 ° C
- Concentrated solutions at room temperature
- Light sensitive reagents stored at appropriate temperature and kept in a *brown bottle* or *cover with foil* or *in a box*.

f) Discard buffers

- Whenever discoloration occurs
- Contamination appears
- Precipitate persists after warming in water bath at 37° C for 20min (or check for salt cyrstals under microscope X100). Salt crytals are large while contaminants are uniform and small

DNA TECHNIQUES

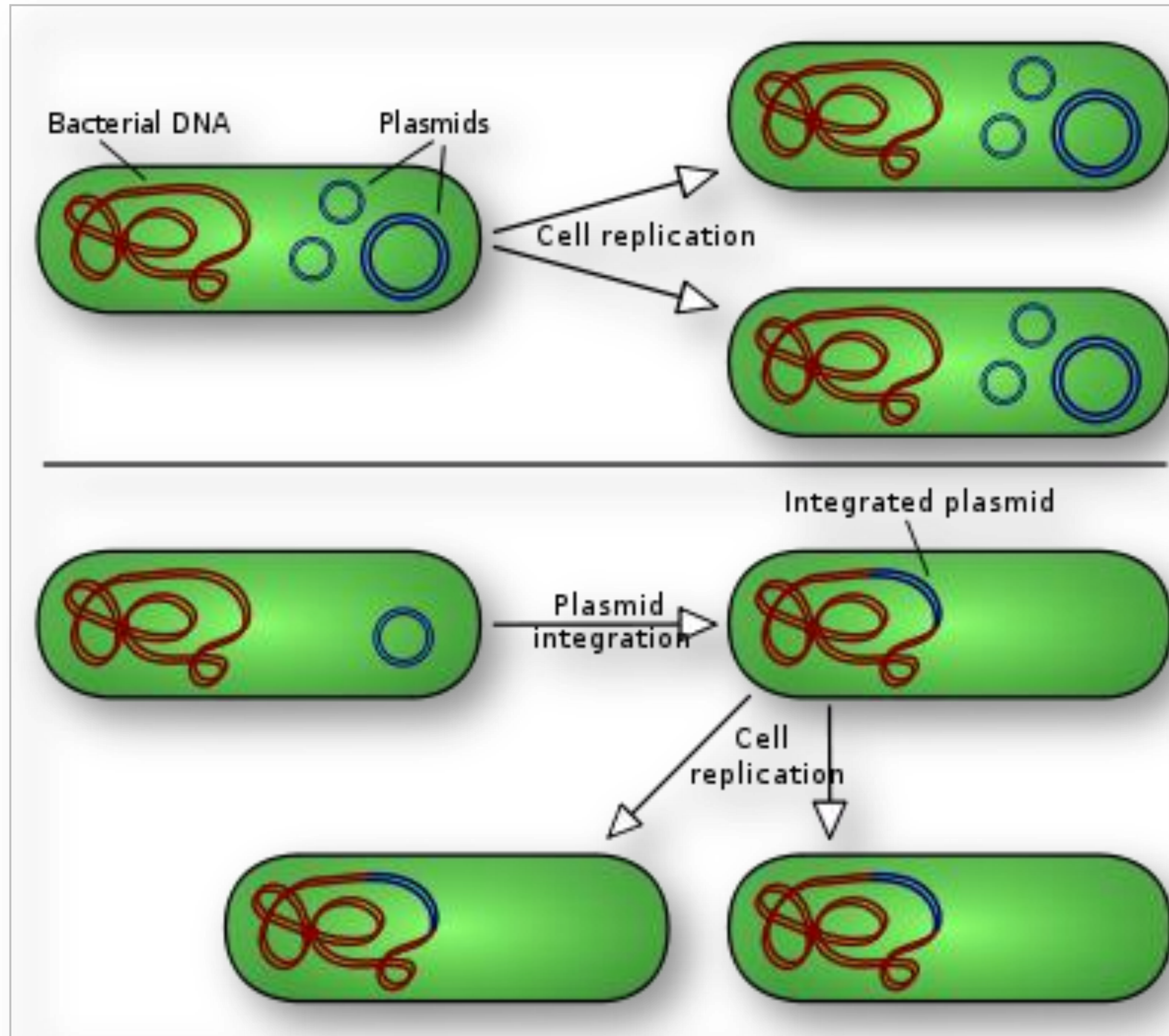
- Plasmid & Plasmid isolation
- DNA restriction digestion
- Ligation (joining)
- Transformation
- Polymerase chain reaction (PCR)
- Agarose gel electrophoresis
- Determination of DNA molecular weight



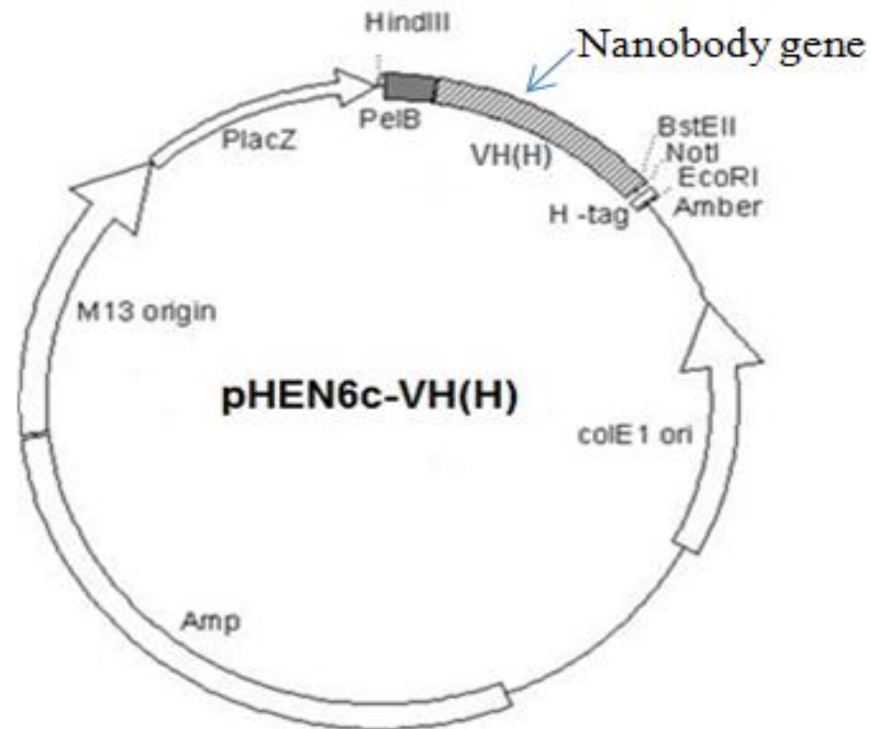
Cloning into a plasmid

a) Plasmid

- Circular molecules of independent extragenomic DNA in bacteria
- Posses one or more vital genes e.g. Antibiotic resistance
- Most have atleast one *ori*-able to replicate independently of the bacterial chromosome (x-some).
- Some plasmids (episomes) insert into the x-some for their replication to occur and excise at a later stage to exist as an independent DNA molecule.

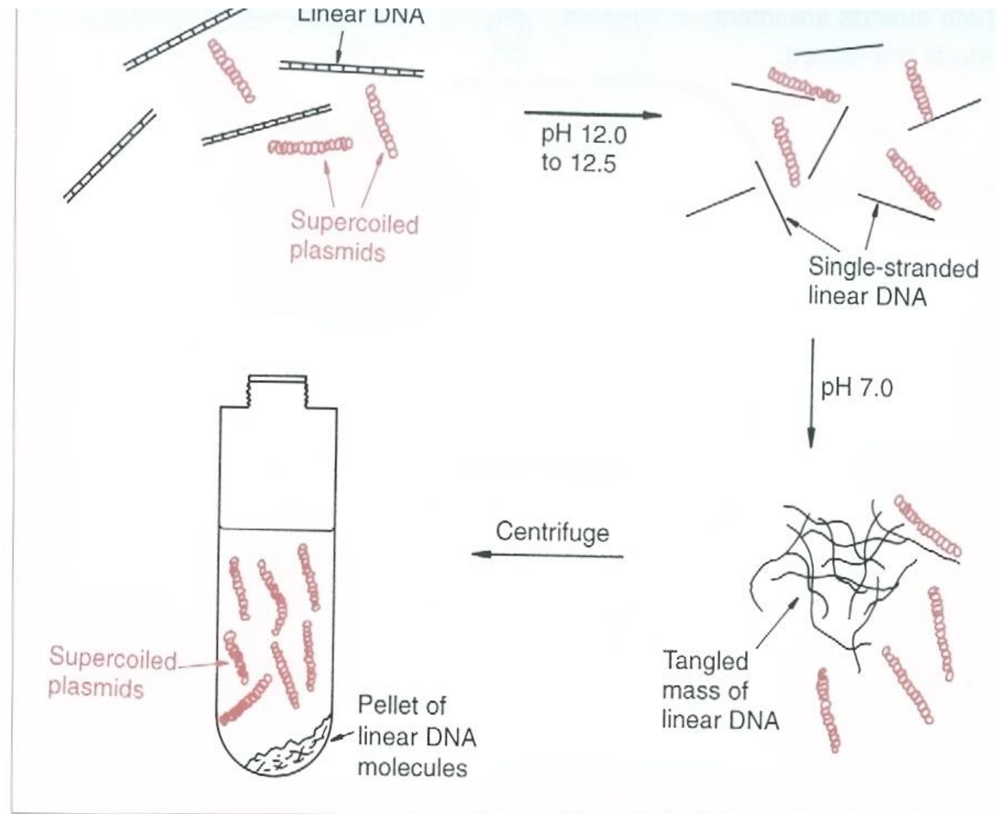


Artificially modified Plasmid



b) Isolation of plasmid DNA

Alkaline denaturation

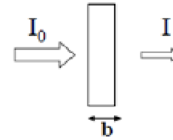


c) Determining Nucleic Acid Concentration & Purity by UV spectroscopy

Principle

Beer Lamberts Law

$A = \varepsilon b c$



1. Turn the spectrophotometer on.
2. Turn on the UV lamp 20 minutes before you will take your readings. The visible light lamps can be used immediately, but the UV lamp takes a while to become steady. The amount of warm-up time needed depends on the lamp and the spectrophotometer.
3. Your sample will be DNA or RNA in water or buffer, with a blank of water or the same buffer. The amount of nucleic acid you add will depend on the source, so ask someone in the lab for a recommendation on the amount of material and the dilution you need.

4. Put the sample and the blank in a matched set of quartz cuvettes.

Only quartz cuvettes, not glass or plastic, will allow accurate readings in the UV range.

5. Set the wavelength to 260 nm.
6. Blank the machine against the water (or blank manually, if only one cuvette at a time can be measured).
7. Read the O.D. at 260.
8. Set the wavelength to 280. Reblank and read the O.D. at 280.
9. Calculate the concentration of the nucleic acid, using the following information:

1 A_{260} unit of double-stranded DNA = 50 µg (50 µg/ml has an O.D. of 1 at 260 nm)

1 A_{260} unit of single-stranded DNA = 37 µg

1 A_{260} unit of single-stranded RNA = 40 µg

$$\text{DNA concentration } (\mu g/ml) = (OD_{260}) \times (\text{dilution factor}) \times \frac{(50\ \mu g\ \text{DNA/ml})}{1\ OD_{260}\ \text{unit}}$$

Example: 10 µl of DNA is added to 390 µl of water, and the O.D. is 0.205.
0.205 x 40 x 50 = 410 µg/ml
The DNA concentration is 410 µg/ml.

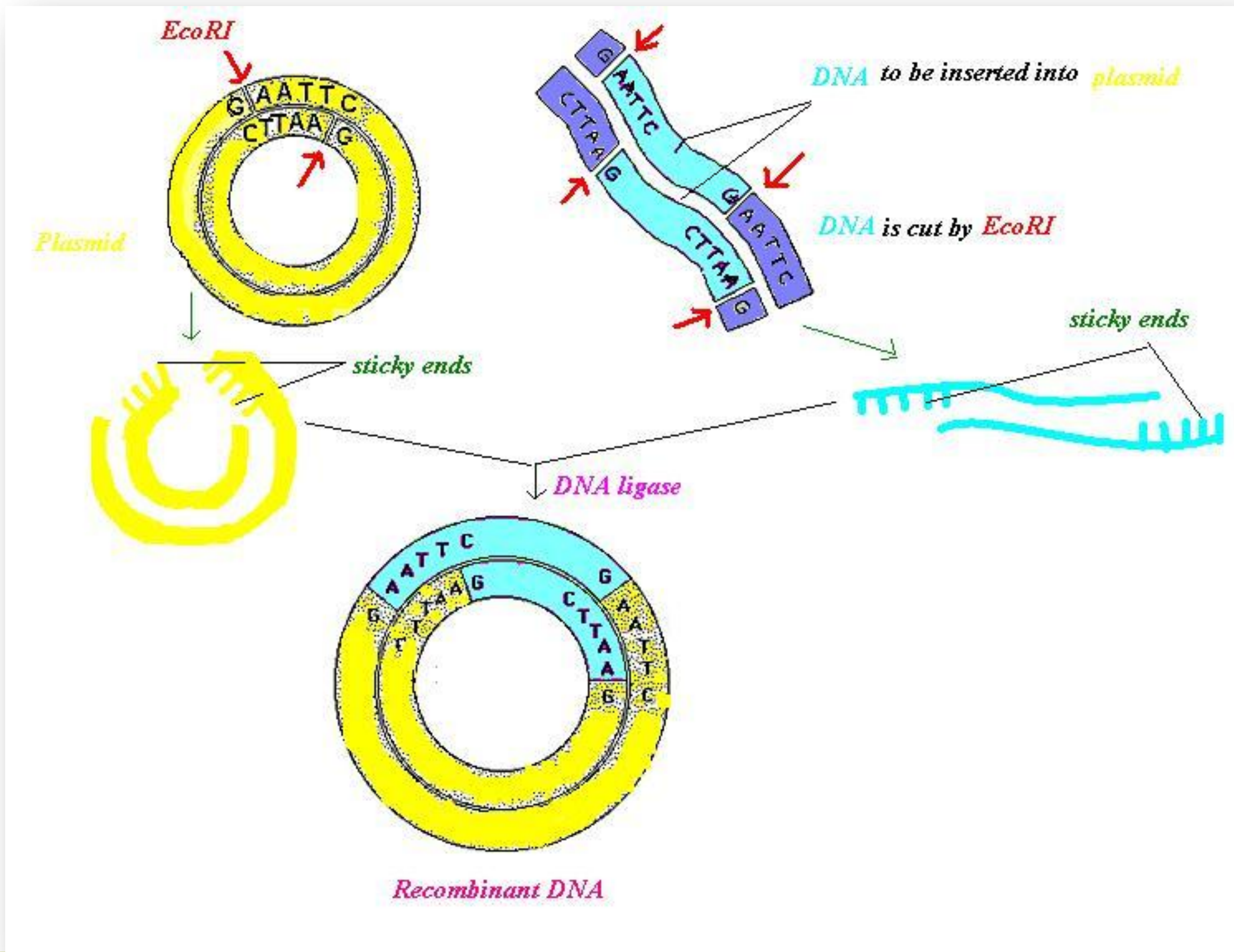
10. Calculate the total yield of your preparation.

Yield = (DNA concentration in µg/ml) x (total volume in ml)
Example: If the 10-µl sample were taken from a 100-µl sample,
410 µg/ml x 0.1 ml = 41 µg or 0.41 mg
Note: If you removed 10 µl from the sample for assay, you would only have 36.9 µg left (41 – 4.1 = 36.9).

11. Estimate the purity of the prep by figuring the 260/280 ratio. The ratio between the readings at 260 nm and 280 nm gives an estimate of the purity of the nucleic acid. Pure preparations of DNA should have a 260/280 ratio of 1.8, RNA a ratio of 2.0. A higher ratio would suggest extraction with phenol:chloroform to remove protein impurities.

Example: If OD_{260} of the DNA prep was .205, and OD_{280} was .114, the 260/280 ratio would be 1.8. Right on the nose!

D) DNA restriction digestion and ligation



E) Transformation

▪ *Transformation*=introduction of foreign DNA into bacterial cell (may also include plant, mammalian cells).

▪May be natural or artificial

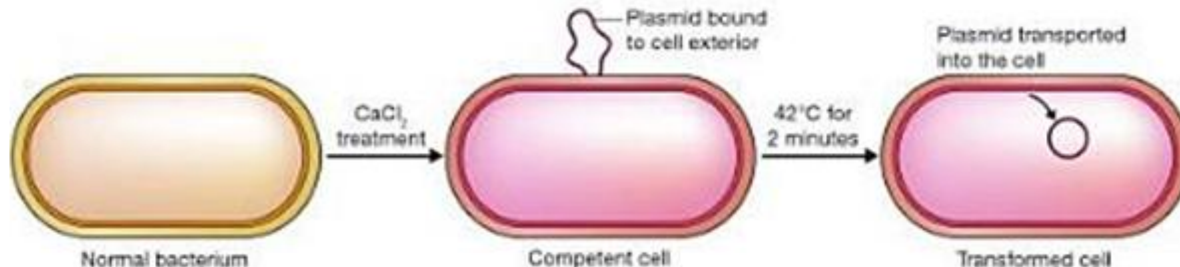
▪Before bacteria are artificially transformed they have to made competent in order to take up DNA

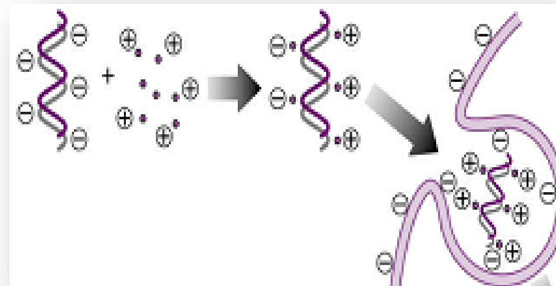
Alternatively, bacteria can acquire foreign DNA through:

▪*Conjugation* ► Mating btn bacteria

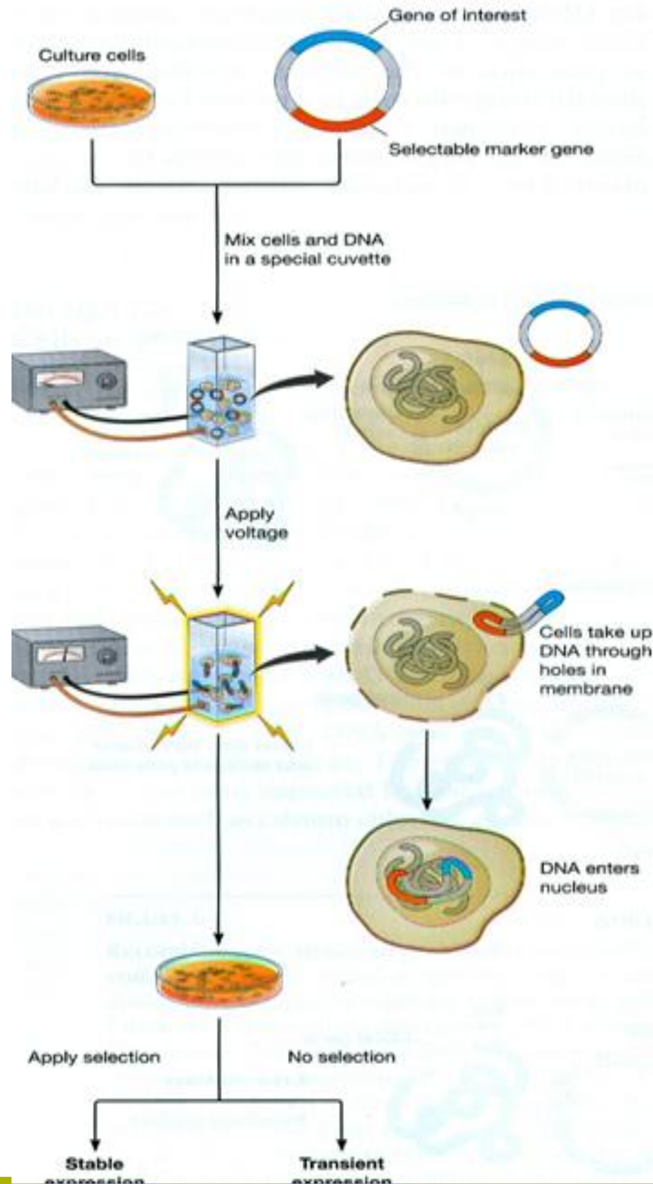
▪ *Transfection* ► Bacteriophage to inject the foreign DNA into cell

1) Chemical transformation





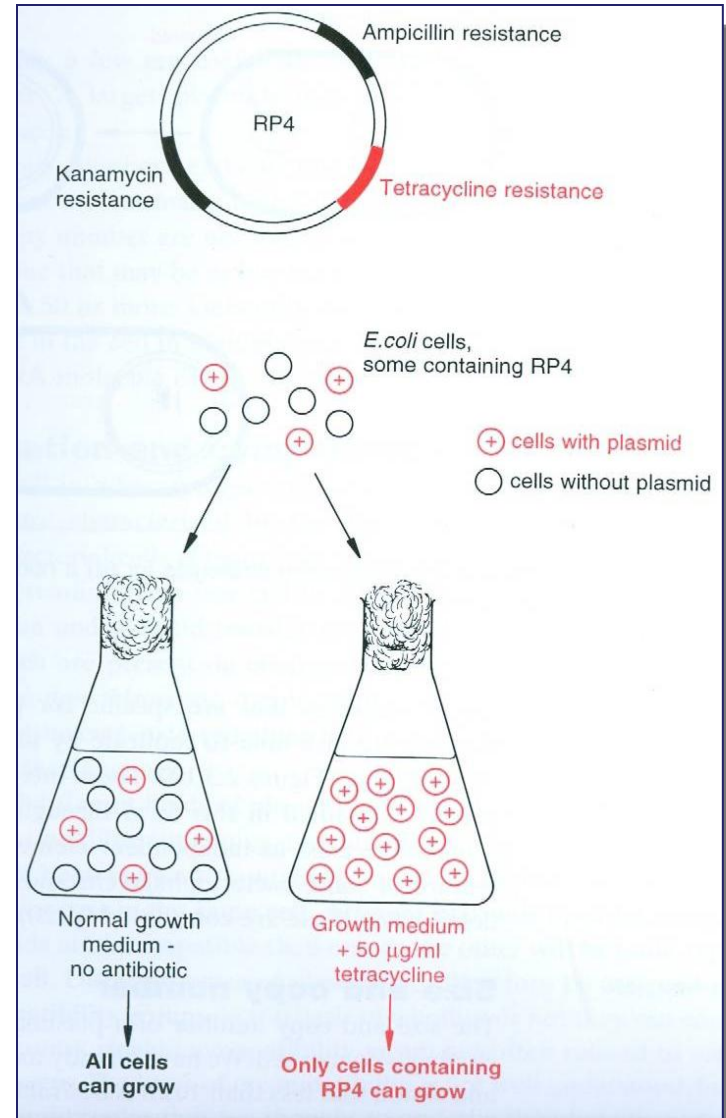
2) Electroporation



Gene transfer by electroporation. Cells are mixed with the DNA to be transfected and placed in a small chamber with electrodes connected to a specialized power supply. A brief electric pulse is discharged across the electrodes, which transiently opens holes in cell membranes. DNA enters the cells, which are removed and plated in fresh medium. The cultures can be harvested for experiments during the transient expression phase, or selection can be applied to isolate stably transfected clones.

F) Selection of transformants (Antibiotic resistance)

- When bacteria are transformed in the laboratory, it acquire new traits from the transformation plasmid.
- These traits are easily identifiable and allow for selection of transformed cells.
- E.g. the bacteria transformed in here acquire resistance to the antibiotic tetracycline.
- Select transformed cells by growing on tetracycline containing growth medium.
- Untransformed bacteria will not grow on this medium.



G) Transformation efficiency

- Measure of the amount of cells within the bacterial culture able to take up DNA molecules.

- Determined experimentally e.g. *to detemine transformation efficiency of an intact plasmid DNA at two different concentrations (1x & 10X)*:

1. Count the average number of colonies growing on the 2 LB:AMP plates.
2. Determine the average amount of DNA that was spread on the plates. Use the examples below to determine the average amount of DNA spread on the plates:
    a. Total DNA supplied in tube = 20 µg.
    b. Volume of transformation solution added to reconstitute = 250 µL.
    c. Concentration of DNA solution = 20 µg / 250 µL (or 0.08 µg/µL).
    d. For the 1X transformation, 10 µL of the DNA solution is added into 250-µL of bacteria resuspended in transformation solution, so the total volume in the tube = 260 µL, and
    e. the total DNA concentration in the tube = 10 µL × 0.08 µg/µL = 0.8 µg DNA/260 µL.

f. After transformation, 100 µL is added to each plate, so the amount of DNA plated = (0.8 µg/µL/260 µL) × 100 µL = 0.3 µg per plate.

g. For the 10x transformation, 100 µL of DNA solution is added into 250 µL of bacteria solution, so the total volume in the tube = 350 µL, and

h. the total DNA concentration = 100 µL × 0.08 µg/µL = 8.0µg/350 µL.

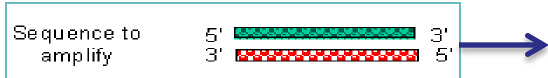
i. After transformation, 100 µL is added to each plate, so the amount of DNA plated = (8.0 µg/350 µL) × 100 µL = 2.3 µg per plate.

3. Calculate transformation efficiency for the 1X and 10X DNA concentrations using the formula below.

$$\text{transformation efficiency} = \frac{\text{total \#of colonies on LB:AMP plate}}{\text{amount of DNA plated(in } \mu\text{g/mL)}}$$

4. Which experiment had greater efficiency?

H) Polymerase chain reaction (PCR)



1. Dena denatur

2. Prime

3. Prime

1) Dena

Cycle 2

1) Denaturation, 2) Primer Annealing, 3) Primer Extensio n

Cycle3

Primer #1

Primer #2

newly made DNA

HindIII

Nanobody gene

PelB

PlacZ

VH(H)

BstEII

NotI

EcoRI

Amber

H -tag

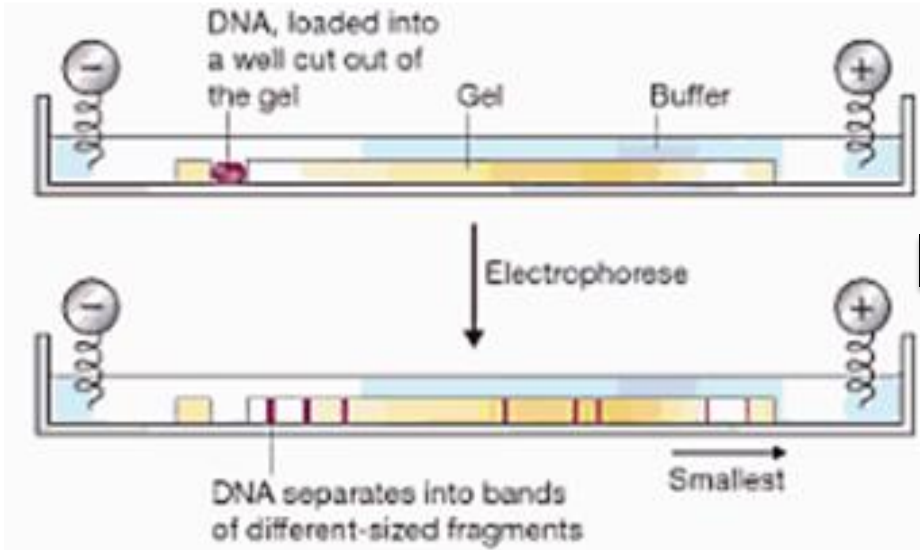
M13 origin

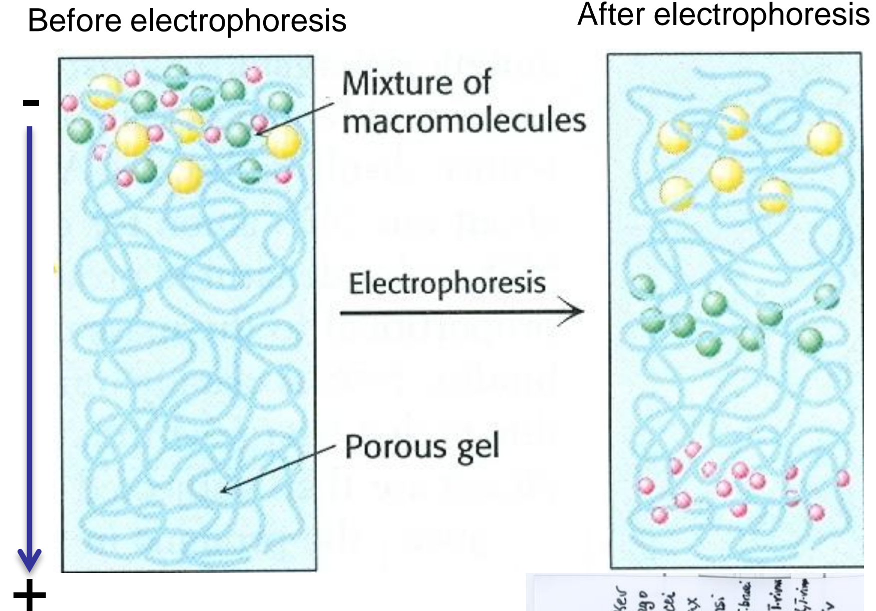
pHEN6c-VH(H)

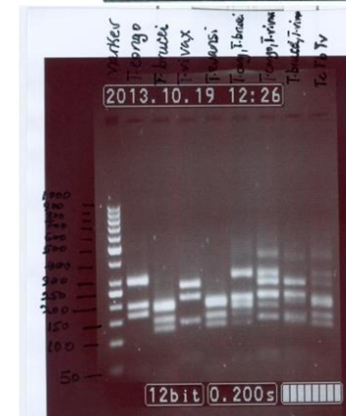
colE1 ori

Amp

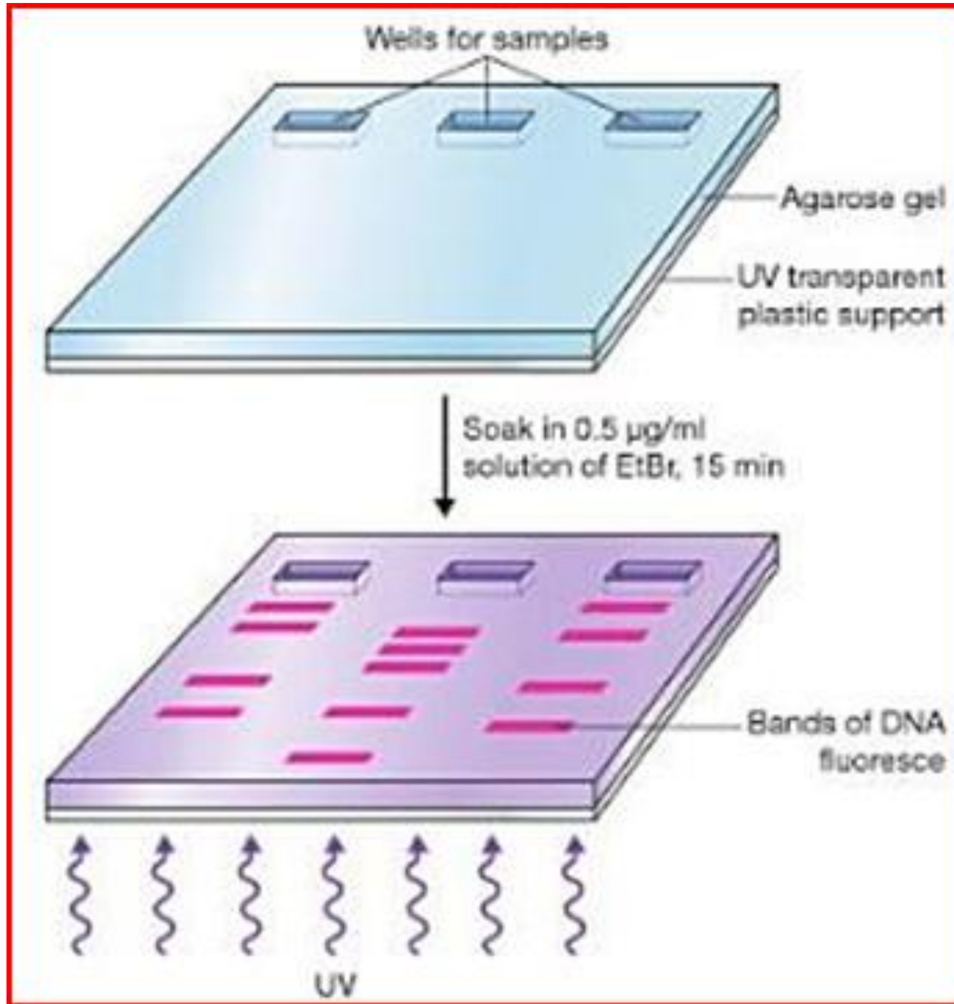
I) Agarose gel electrophoresis to detect DNA

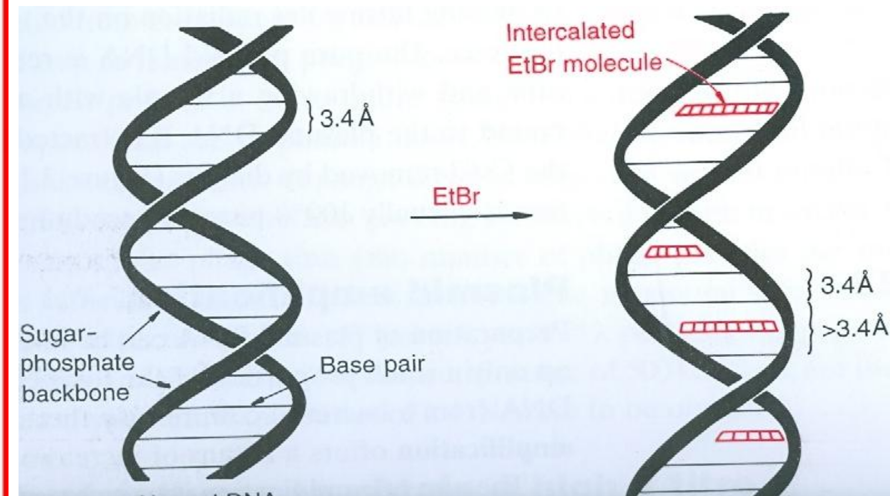




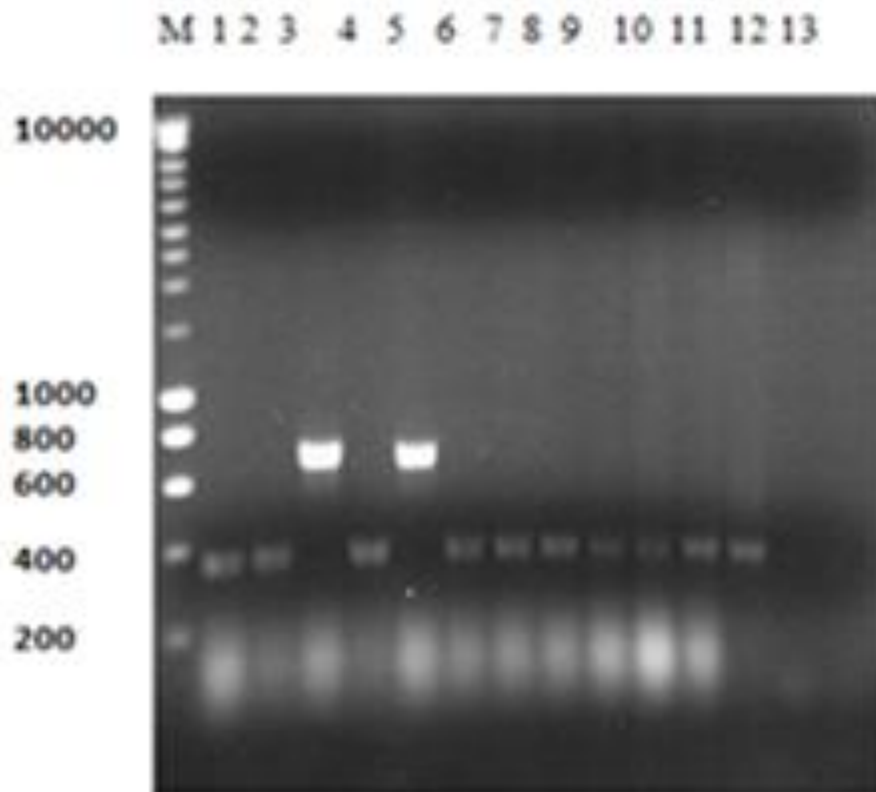


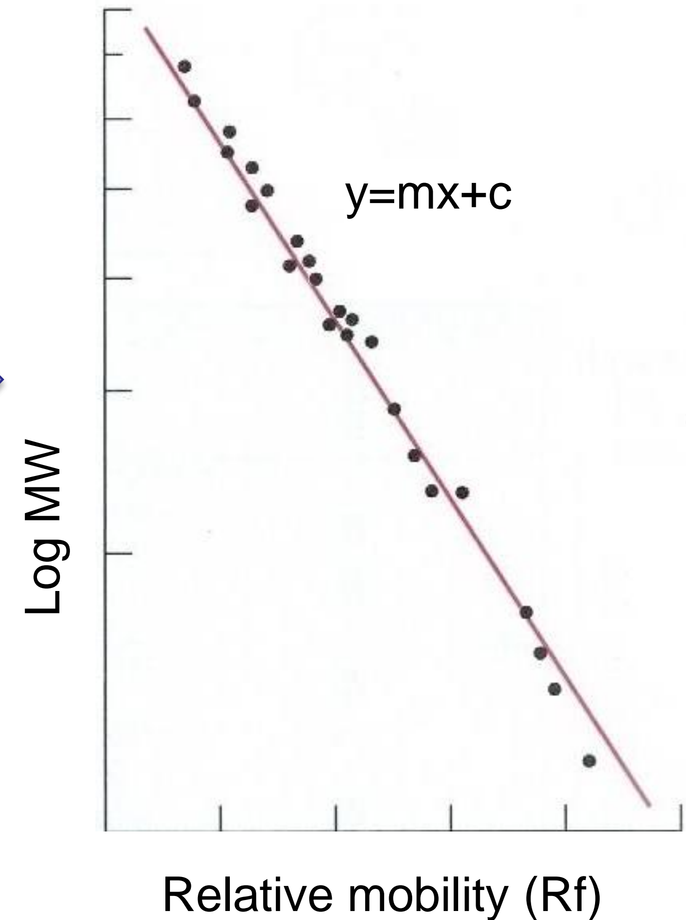
J) UV visualization of DNA bands (ethidium bromide)





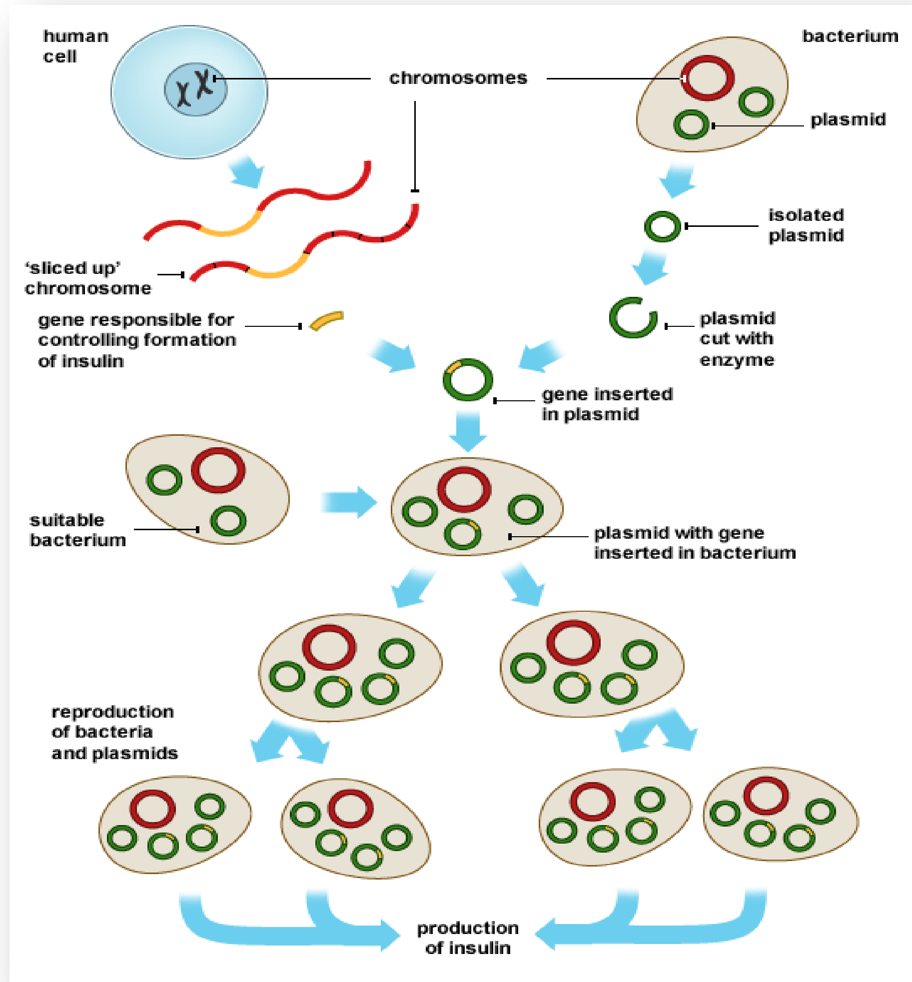
K) Determination of DNA molecular weight



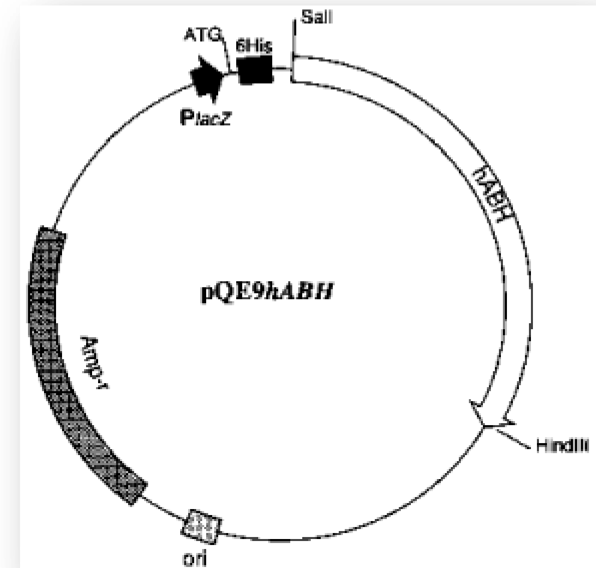


$$Rf = \frac{\text{Distance of DNA migration from the origin}}{\text{Distance of migration of dye from the origin}}$$

a) Expression of recombinant protein



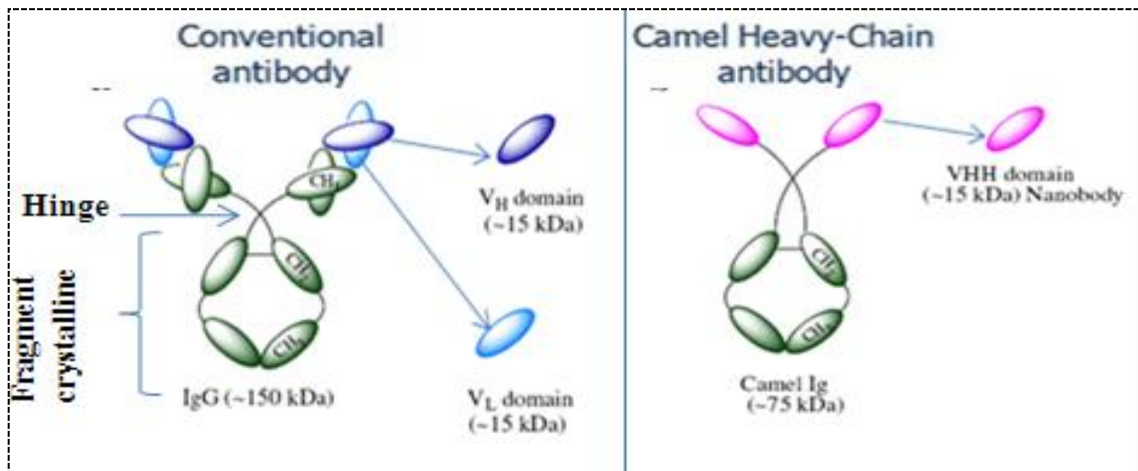
- **Is it possible to manipulate bacteria to become protein production factories?**
- **Yes, already we have performed major manipulation in the preceding section. Here we start protein production**

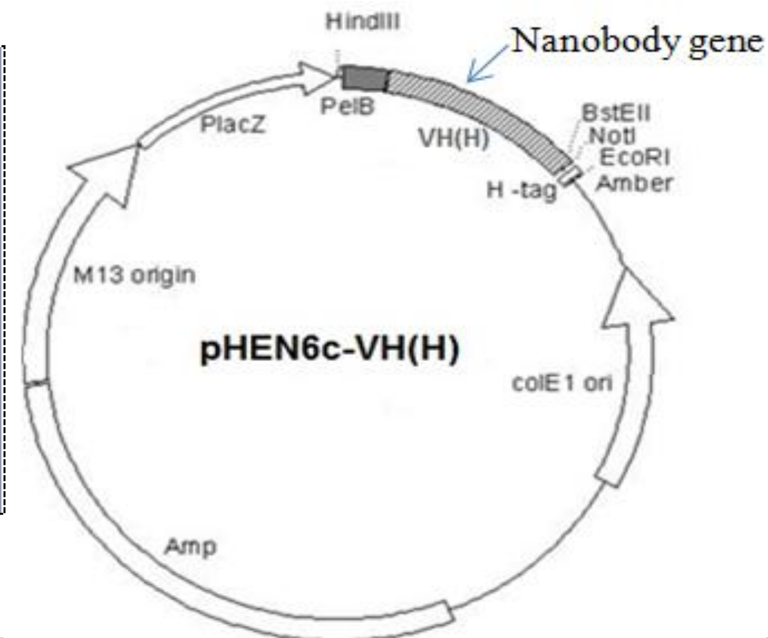


E.g. Expression of Nanobody

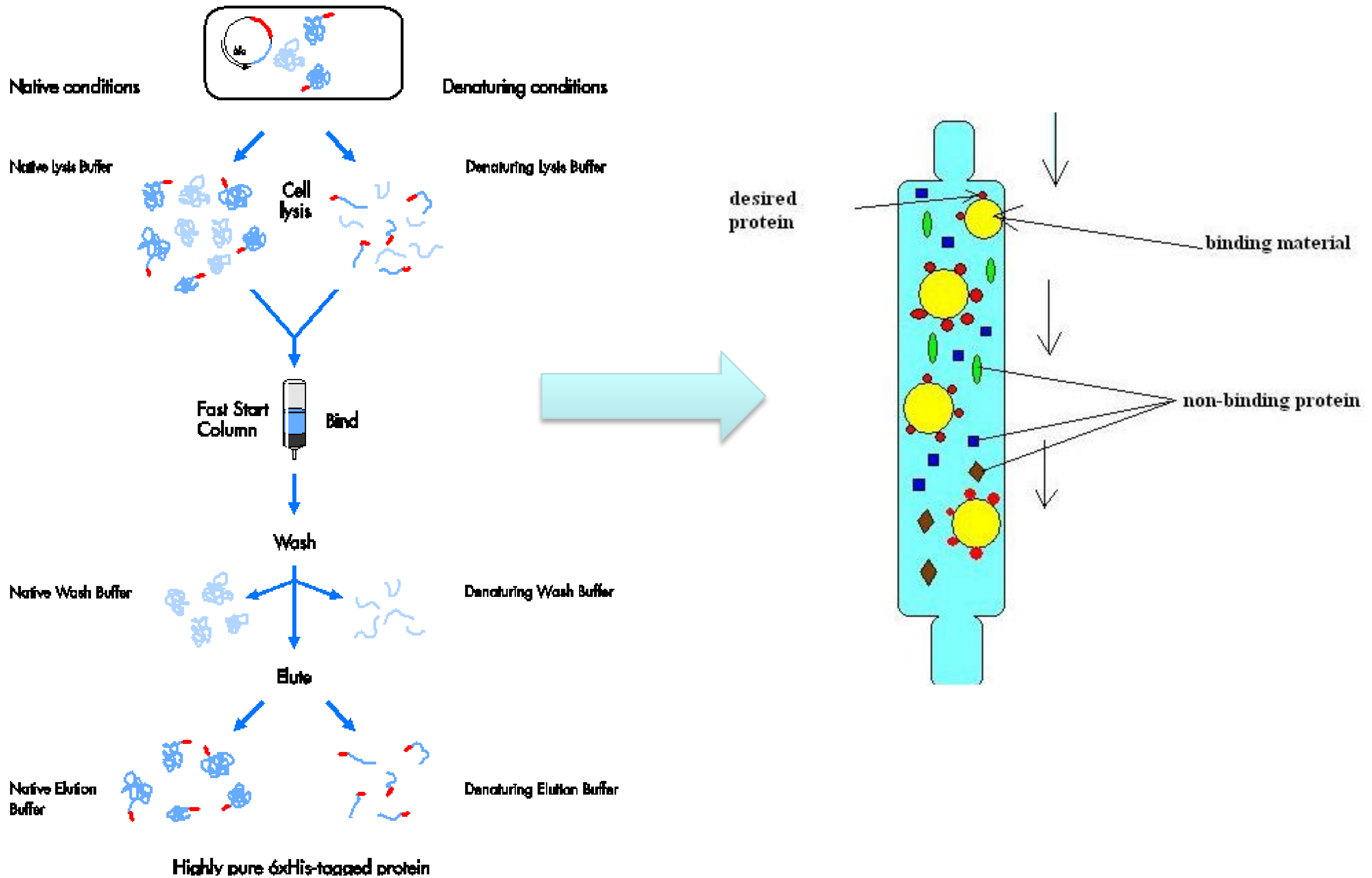
•How cool would it be to take advantage of these microorganism's sophisticated makeup, short doubling times and cheap growth media to mass produce medically and commercially useful proteins?

•All of these are possible with a few simple genetic manipulations





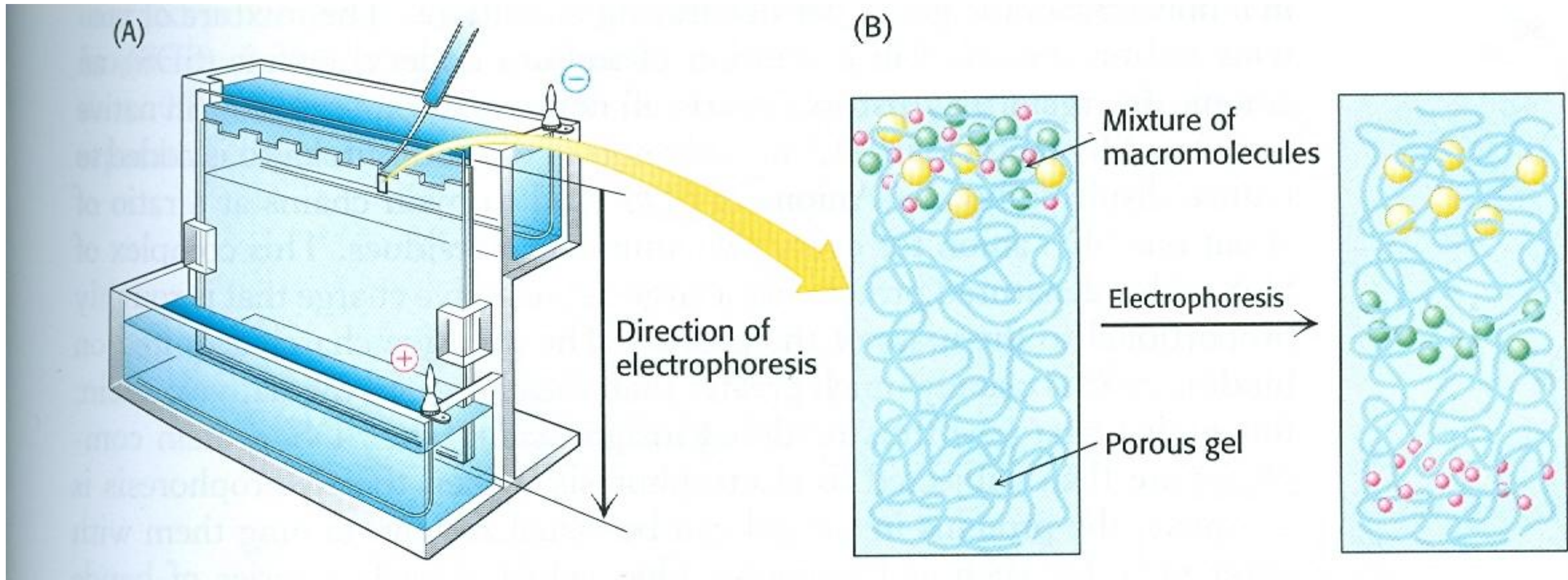
b) Protein expression, extraction and purification



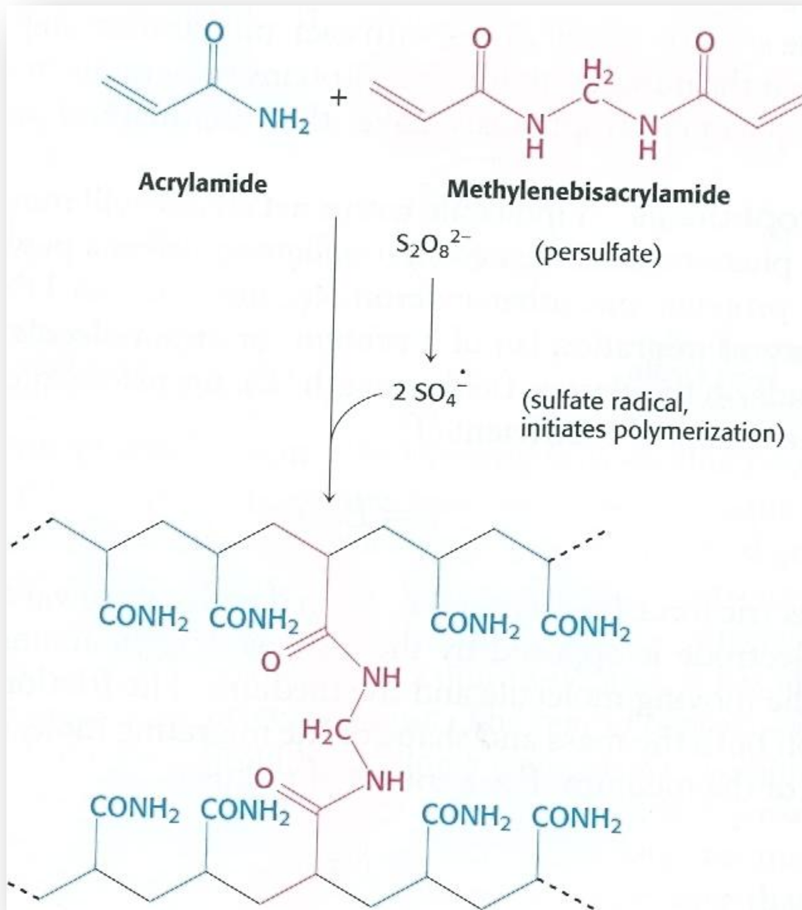
c) Protein detection

I) Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS PAGE)

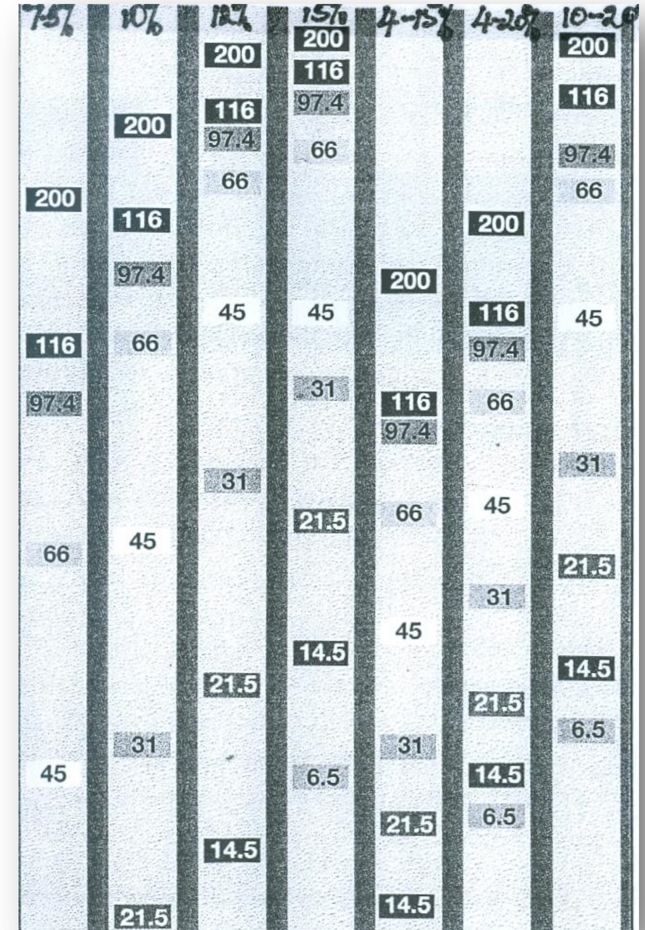
Principle



Components of SDS-PAGE and mechanism of polymerization



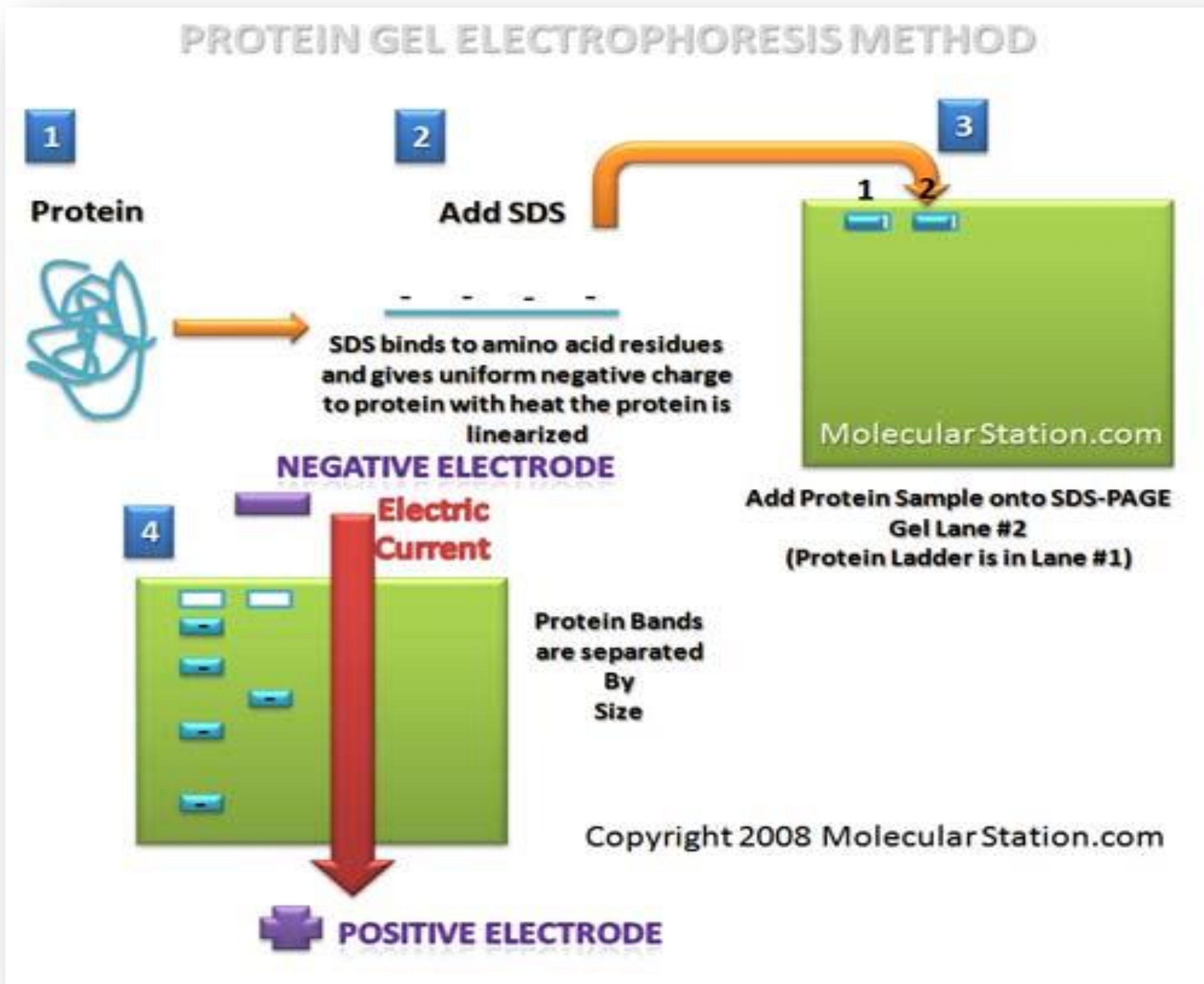
Relative protein migration vs SDS-PAGE concentration



Protein molecular-weight markers. The positions of proteins of 200, 116, 97.4, 66, 45, 21.5, 14.5, and 6.5 kD are shown after electrophoresis through gels of different acrylamide concentrations, using Tris-glycine buffer. (Redrawn,

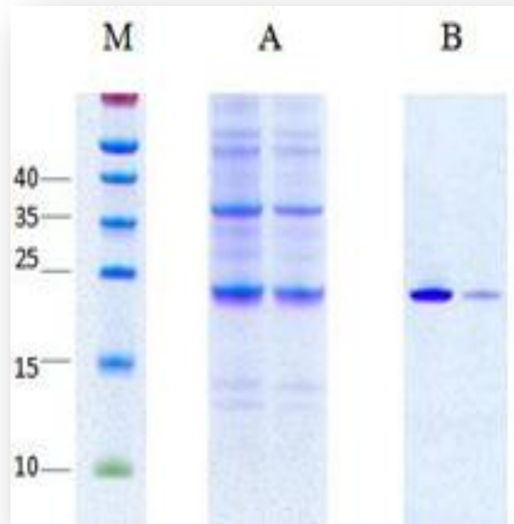
Run 25–30mA (200v) Stop when dye front Reaches the bottom of the gel

SDS-PAGE Steps



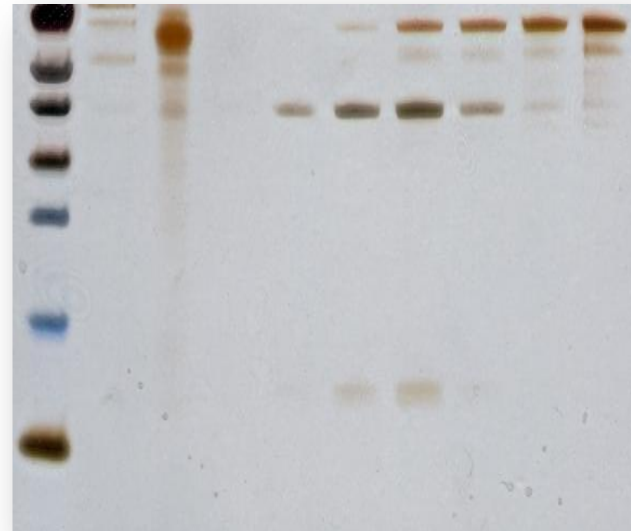
VISUALIZING PROTEIN BANDS

Coomassie blue



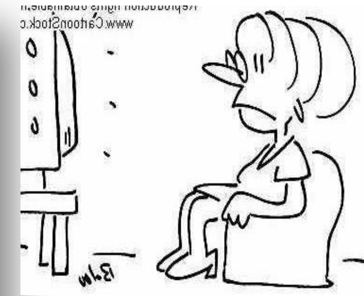
Good for basic protein

Silver staining (more sensitive)



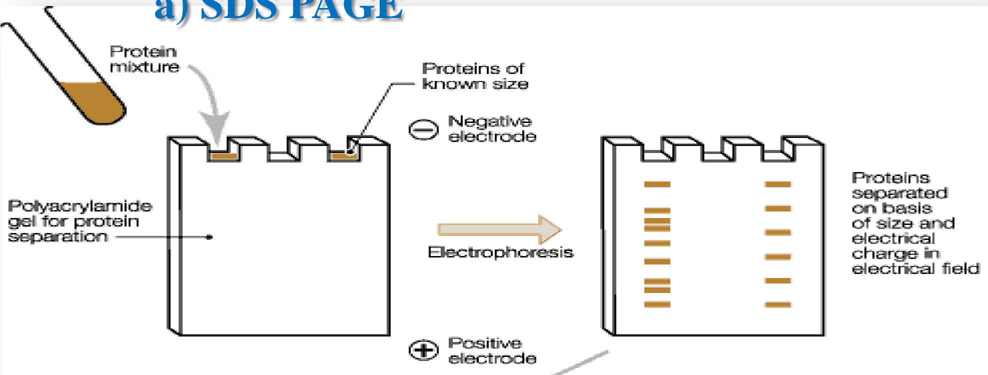
Good for acidic protein

II) Western blot to detect protein

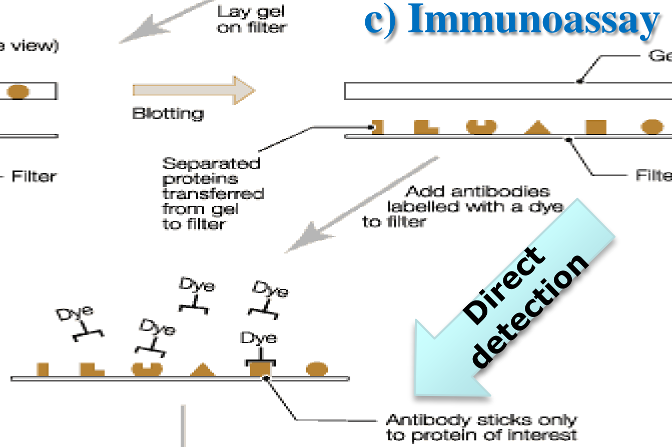


Which of these bands is my protein of interest?
Is there Ab specific to my protein? Yes, then do WB

a) SDS PAGE



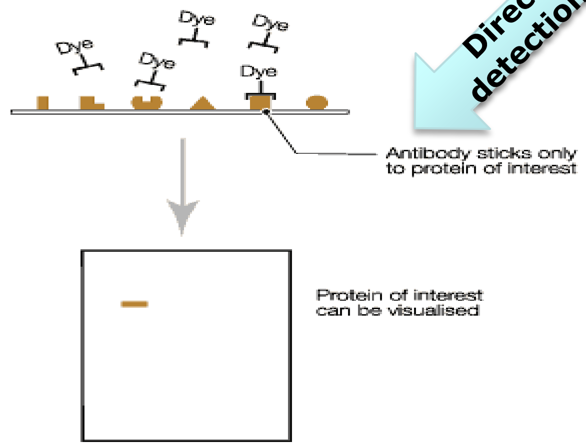
b) Blotting

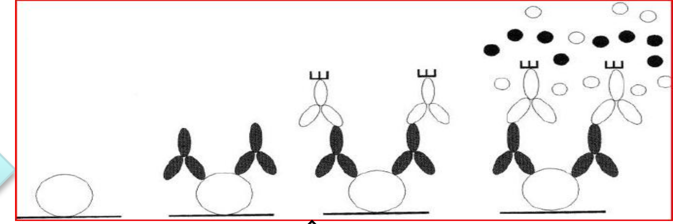


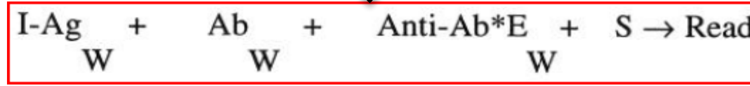
c) Immunoassay

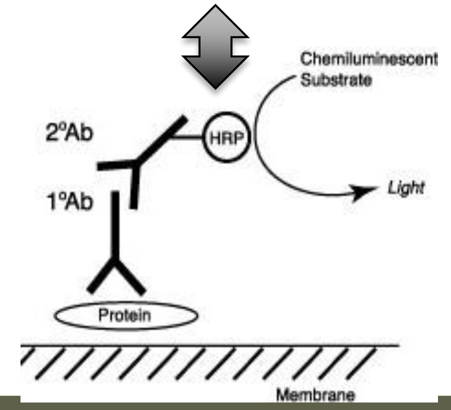
Indirect detection

Direct detection

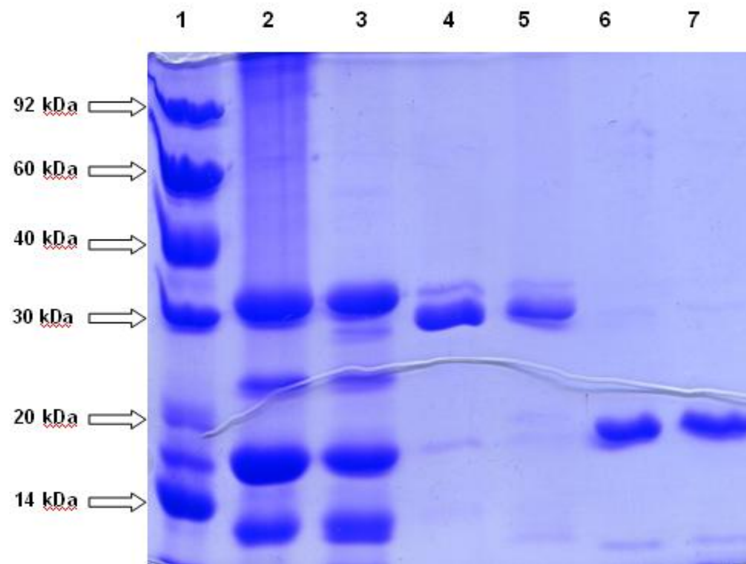








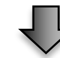
d) Calculating MW of protein after SDS-PAGE

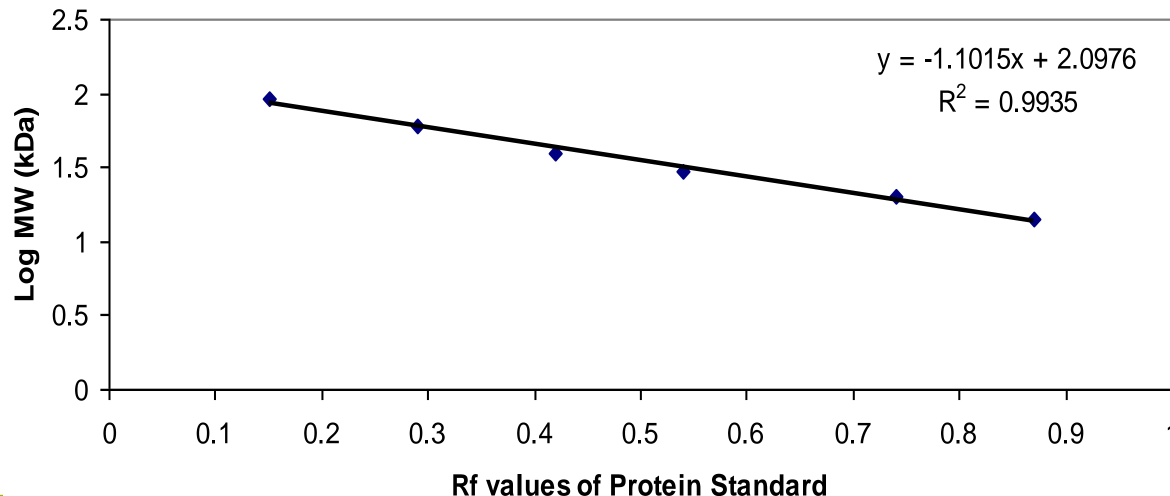


(Rf) = Distance of DNA migration from the origin / Distance of migration of dye from the origin

Protein marker	1	2	3	4	5	6
Rf (X)	0.15	0.29	0.42	0.54	0.74	0.87
MW	92	60	40	30	20	14
Log MW (Y)	1.96	1.78	1.6	1.48	1.3	1.15

R_f value of sample = 0.56, MW ?





Calculation

Y = -1.1015x + 2.0976

= -1.1015 (0.56) + 2.0976

= 1.48

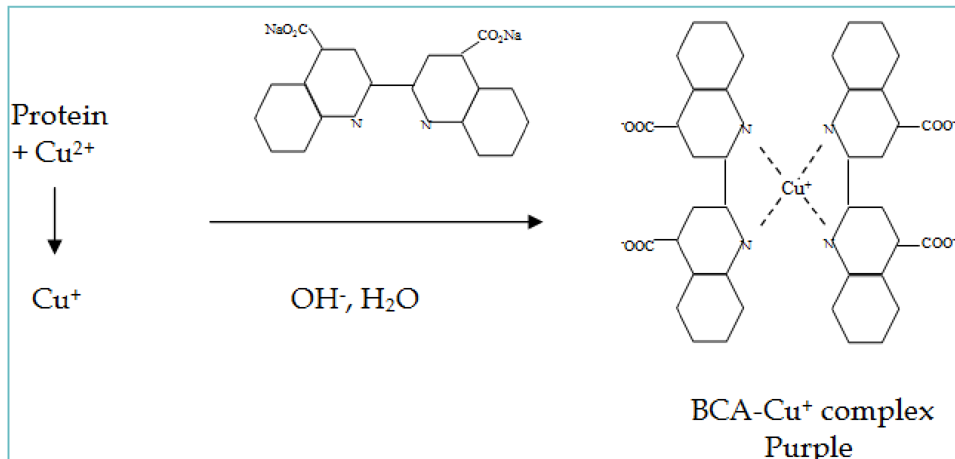
Therefore, the molecular weight of the sample equals to the antilog of 1.48 = <u>30.3kDa</u>

e) Determination of protein concentration (BCA)

How it works. Copper sulfate, added to an alkaline solution of BCA (bicinchonic acid), gives an apple-green colored complex. When this solution is added to a protein solution, the Cu^{++} ions are converted to Cu^{+} by interaction with the peptide bonds of the protein, changing the color of the complex to purple with an absorbance maximum of 562 nm. Pierce makes a BCA assay reagent.

Advantages. Fast, sensitive, accurate.

Disadvantages. Subject to interference by agents such as detergents and organic solvents. Time-dependent, color develops for 24 hours.



I) BCA Steps

- From the stock solution of 2 mg/ml of BSA prepare 1.8 mg/ml, 1.6 mg/ml, 1.4 mg/ml, 1.2 mg/ml, 1.0 mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml and 0.2 mg/ml.

- Prepare 2-fold serial dilutions of sample as indicated in the diagram of microplate

- Fill wells of a flat-bottom micro-titer plate by pipetting 10 µl of each & blank well filled with PBS

- Mixing reagents A and B in a 50:1 proportion

- Incubate plate at 37°C for 30 mins or at RT for 2h

- Read the $\textbf{OD}_{595}$**nm** in a micro-titer plate reader

II) Plotting and calculation of protein concentration

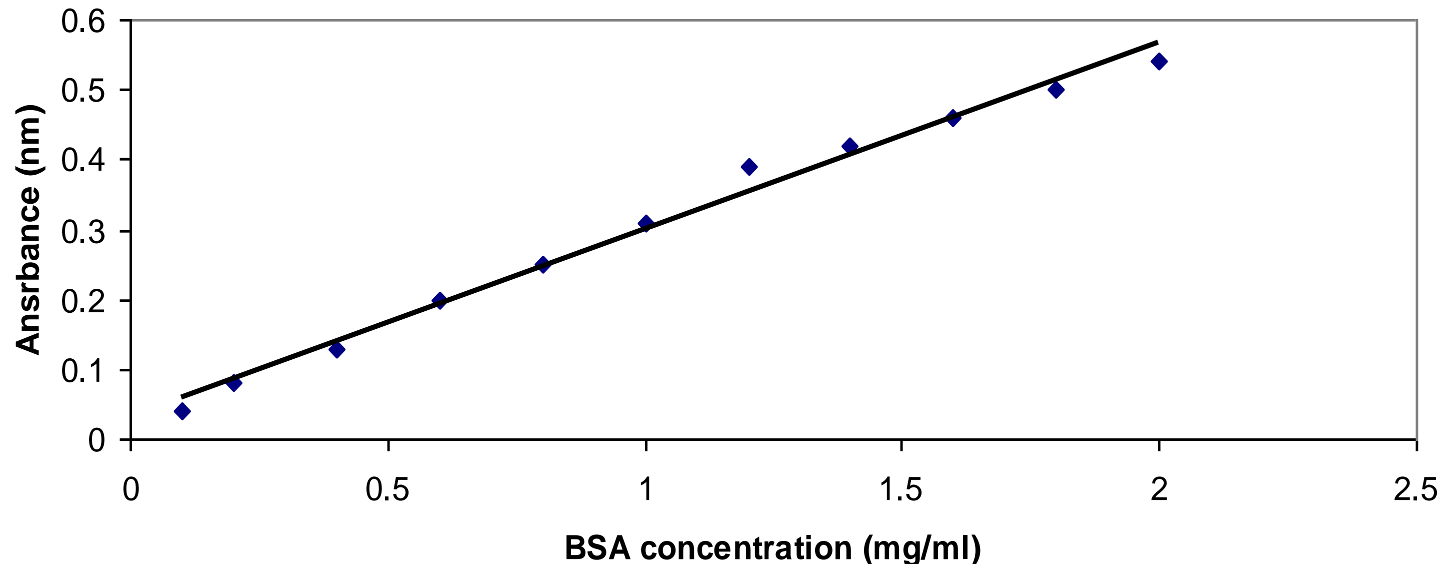
BSA mg/ml: rows A–B; 2-fold diluted sample: rows C–D

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	Blank	
B	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	Blank	
C	Conc.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Blank	
D	Conc.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Blank	
E												
F												
G												
H												

BSA	2	1.8	1.6	1.4	1.2	1	0.8	0.6	0.4	0.2	0.1
Mean A_{595} - Blank	0.54	0.5	0.46	0.42	0.39	0.31	0.25	0.2	0.13	0.08	0.04

Sample dilution	Conc.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Mean A_{595} - Blank	2.18	1.59	0.97	0.52	0.31	0.18	0.11	0.08	0.02	0.01

Plotting and calculation of protein concentration



Absorbance at 1/8 dilution (0.52 nm) was used to calculate protein concentration in the sample because it is the first one within range of standard.

y = 0.267x + 0.0324

0.52 = 0.267x + 0.0324

0.4876 = 0.267x

x = 1.826

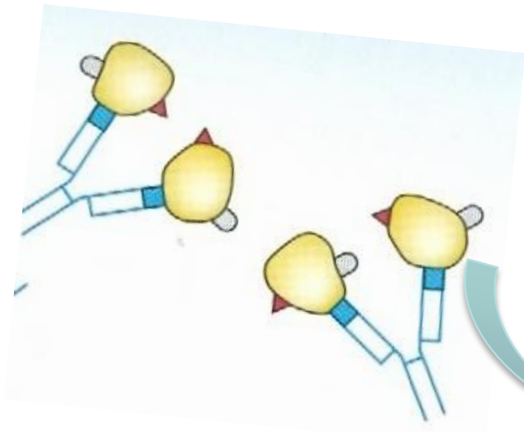
Concentration at 1/8 is 1.826 mg/ml, then concentration of undiluted protein equals to

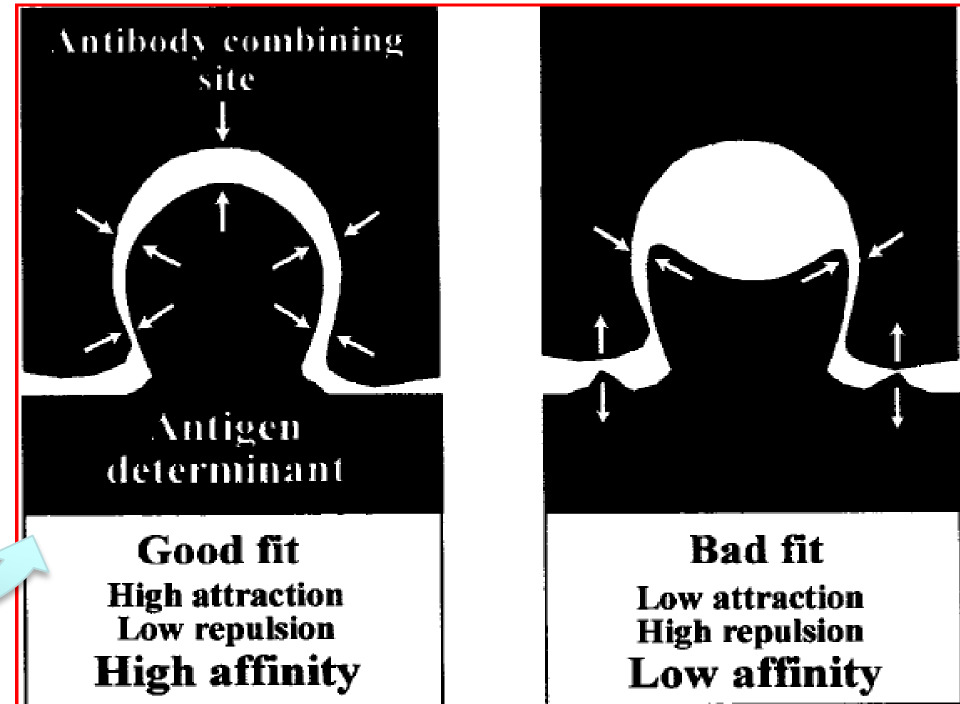
8 (i.e. dilution factor) x 1.826 = 14.608mg/ml

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

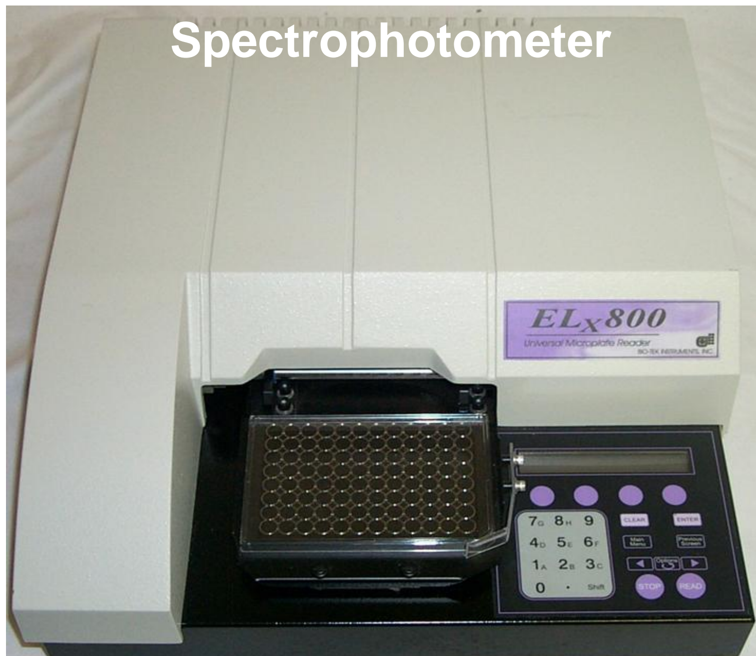
- An immunoassay tool used in research and biomedical sciences.

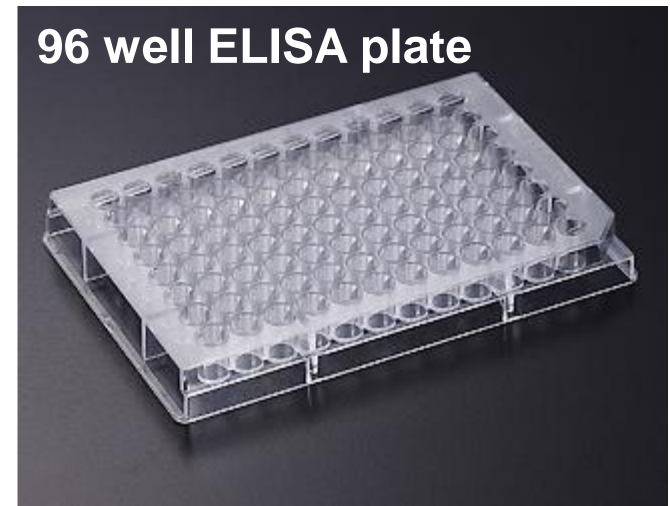
- Ag-Ab interaction





Requirements for solid-phase ELISA





- Antigen (AP/HRP labeled or unlabeled)
- Blocking solution (milk, BSA, Casein etc..), wash solution
- Antibodies (I° or II°), may also be labeled (conjugated) or unlabeled
- Chromogenic substrate (TMB, AP, OPD, ABTS, etc..)

Basic ELISA methods

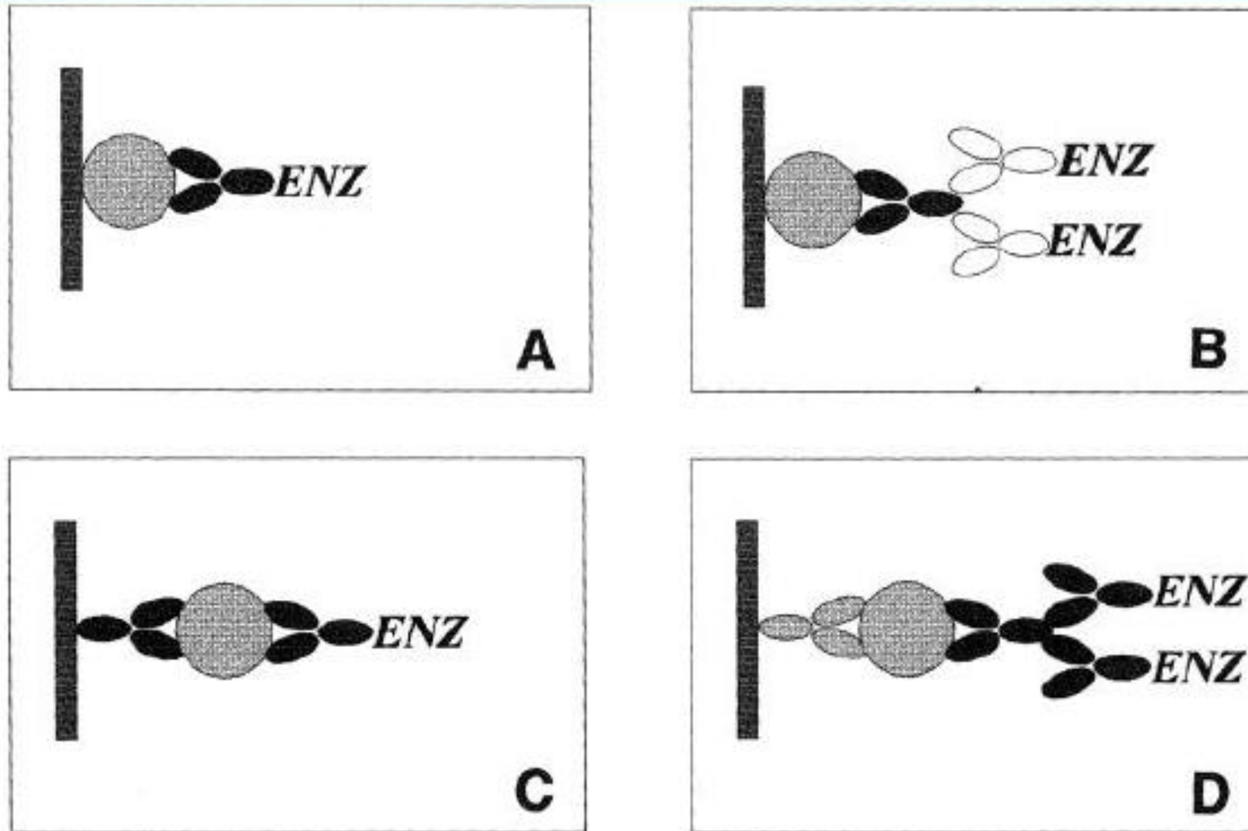
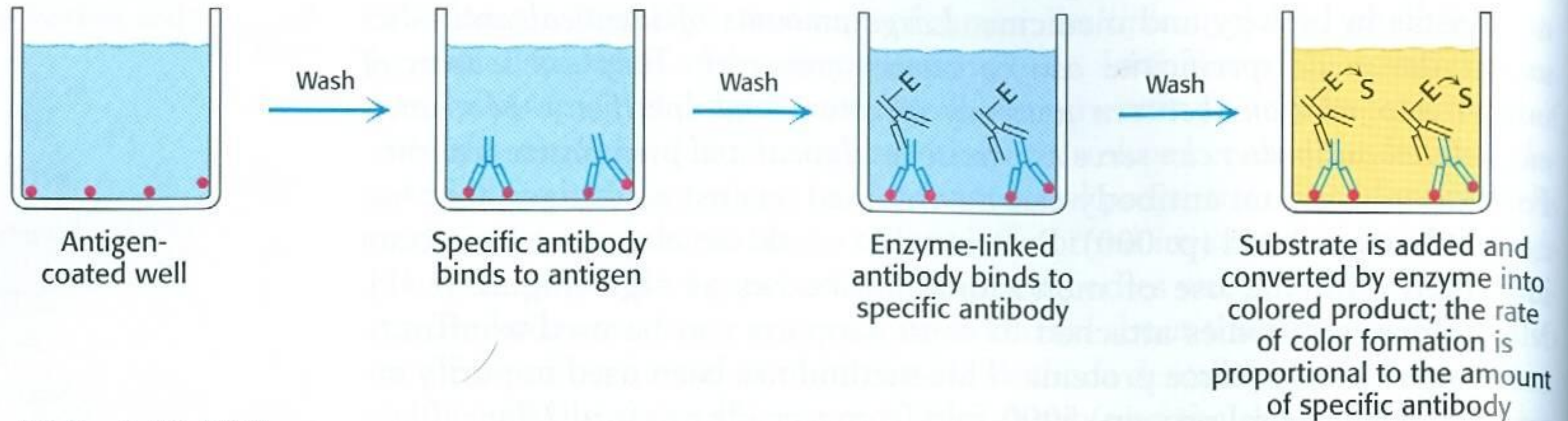
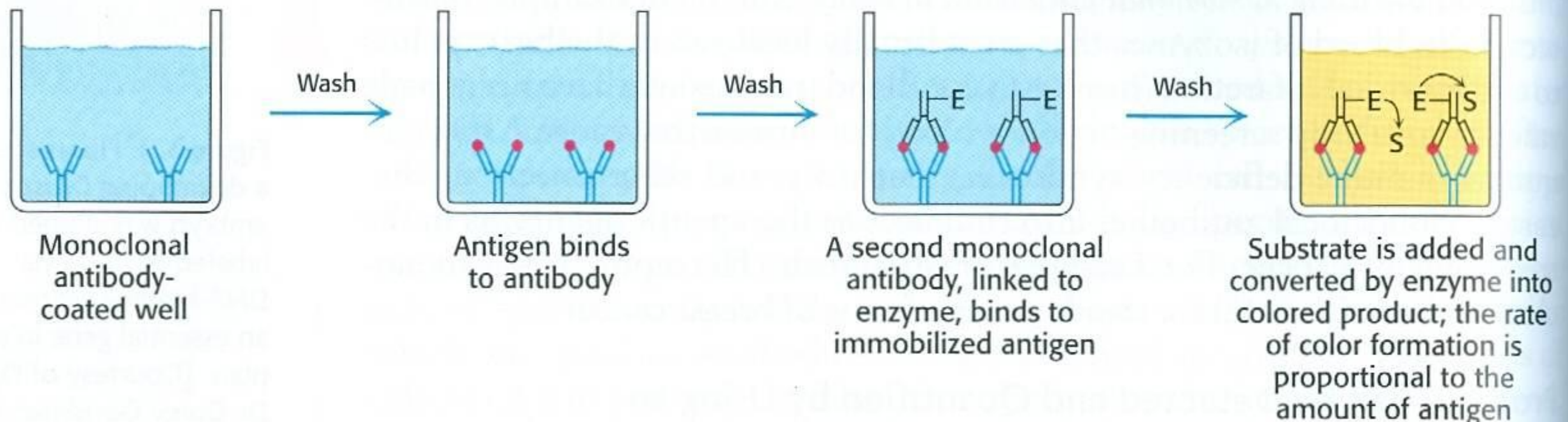


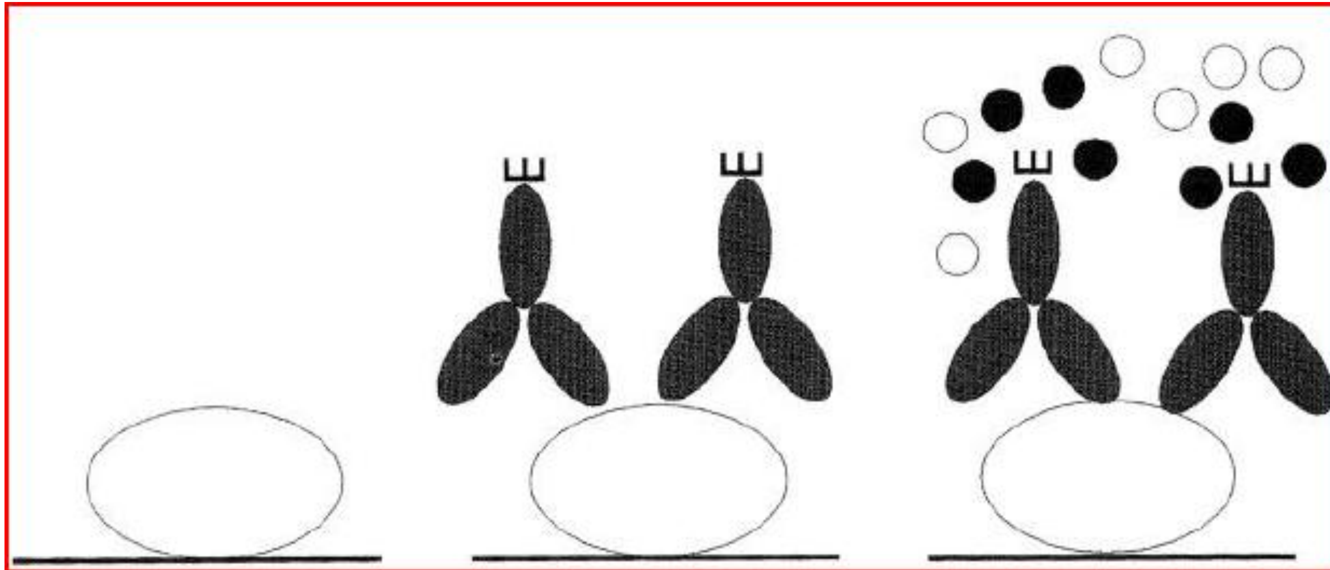
Fig. 11. Basic ELISA methods. (**A**) Direct, (**B**) Indirect, (**C**) Sandwich (direct), (**D**) Sandwich (indirect)

Steps of Indirect & sandwich ELISA



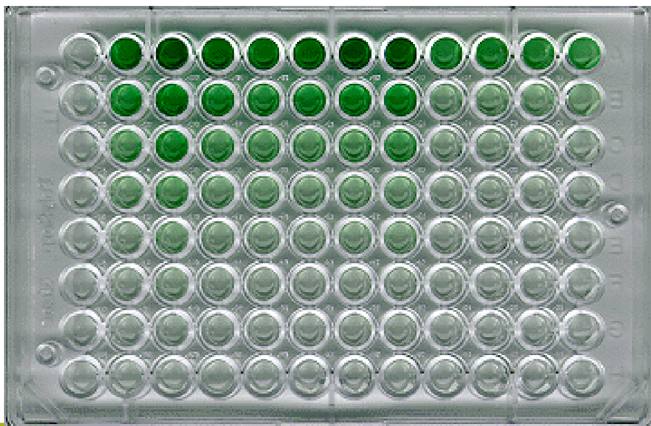


Steps of a direct solid-phase ELISA



Stages in ELISA

1. The adsorption of antigen or antibody to the plastic solid-phase.
2. The addition of the test sample and subsequent reagents.
3. The incubation of reactants.
4. The separation of bound and free reactants by washing.
5. The addition of enzyme-labeled reagent.
6. The addition of enzyme detection system (color development).
7. The visual or spectrophotometric reading of the assay.



TMB or ABTS
catalyzed by HRP enzyme=
product bluish-greenish color

Guide to pipetting

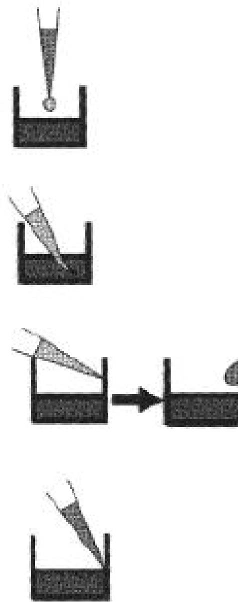
ONLY USE FIRST STOP!

DO NOT DRIP!

DO NOT PRESS HARD INTO WELL!

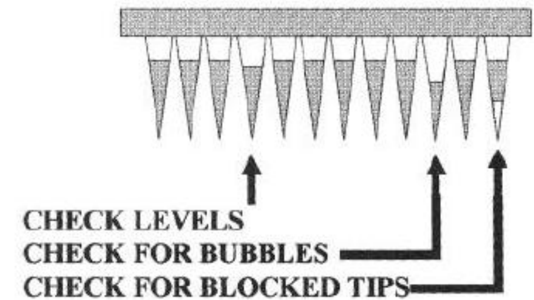
DO NOT USE TOO ACUTE AN ANGLE!

MAKE SURE TIP TOUCHES
SIDE OF WELL AND LIQUID



MULTI-CHANNEL PIPETTING

PUSH TIPS ON TIGHTLY



FILL TO FIRST STOP
EMPTY TO FIRST STOP

HOLD BUTTON DOWN BETWEEN PLATE AND
RESERVOIR WHEN RE-FILLING PLATE

Possible mishaps with coated antibody

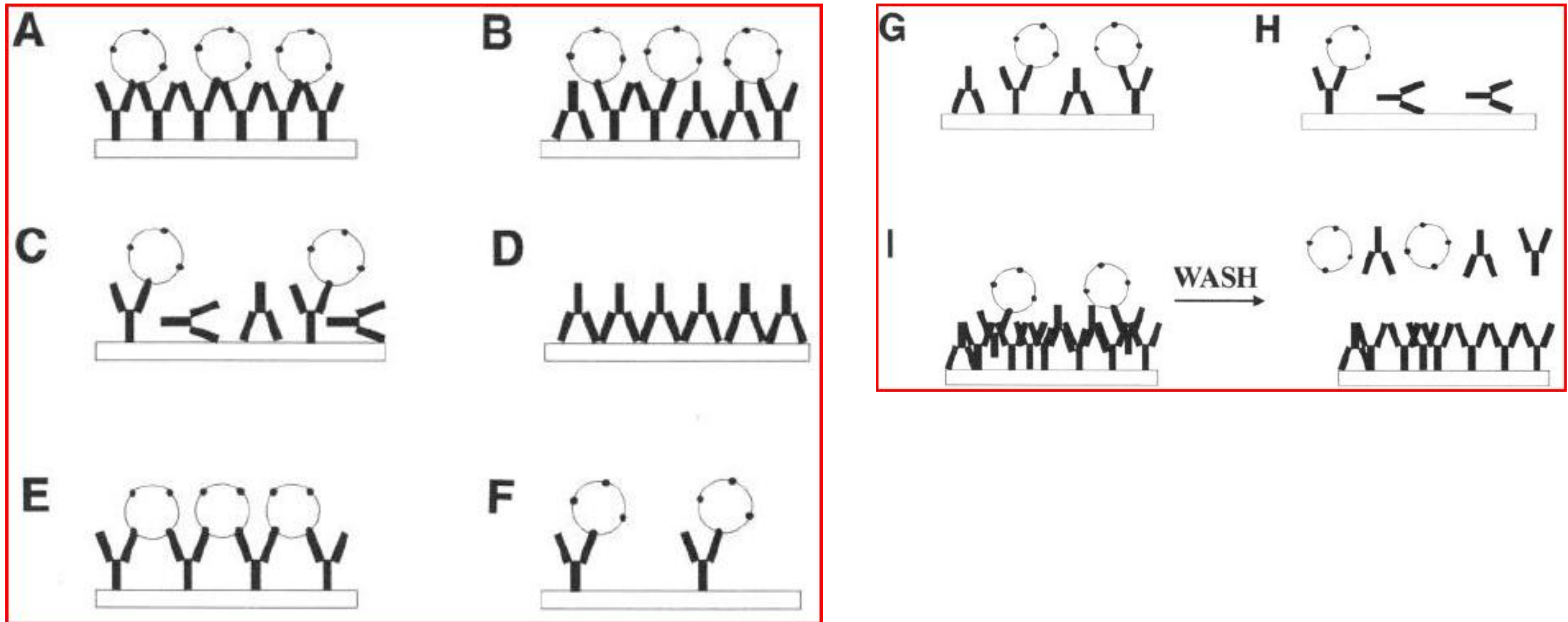


Fig. 1. Effects on antibodies of coating. **(A)** Antibody molecules packed evenly, orientation Fc on plate, monovalent interaction of multivalent Ag. **(B)** Antibody molecules packed evenly, orientation Fc and Fab on plate, monovalent binding of multivalent Ag. **(C)** Antibody binding in all orientations, monovalent binding of multivalent Ag. **(D)** Antibody binding via Fab, no binding of Ag. **(E)** Antibody spaced with orientation to allow bivalent interaction between adjacent antibody molecules. **(F)** Antibody spaced too widely to allow adjacent molecules to bind bivalently via Fc. **(G)** As in (E) except that orientation is via Fc or Fab. **(H)** More extreme case of (C) with less antibody and more molecules inactive because of their orientation. **(I)** Multilayered binding in excess leads to binding, but elution on washing.

THANKYOU

&

WISHING YOU A SUCCESSFUL PRACTICAL