Quantification of Protein

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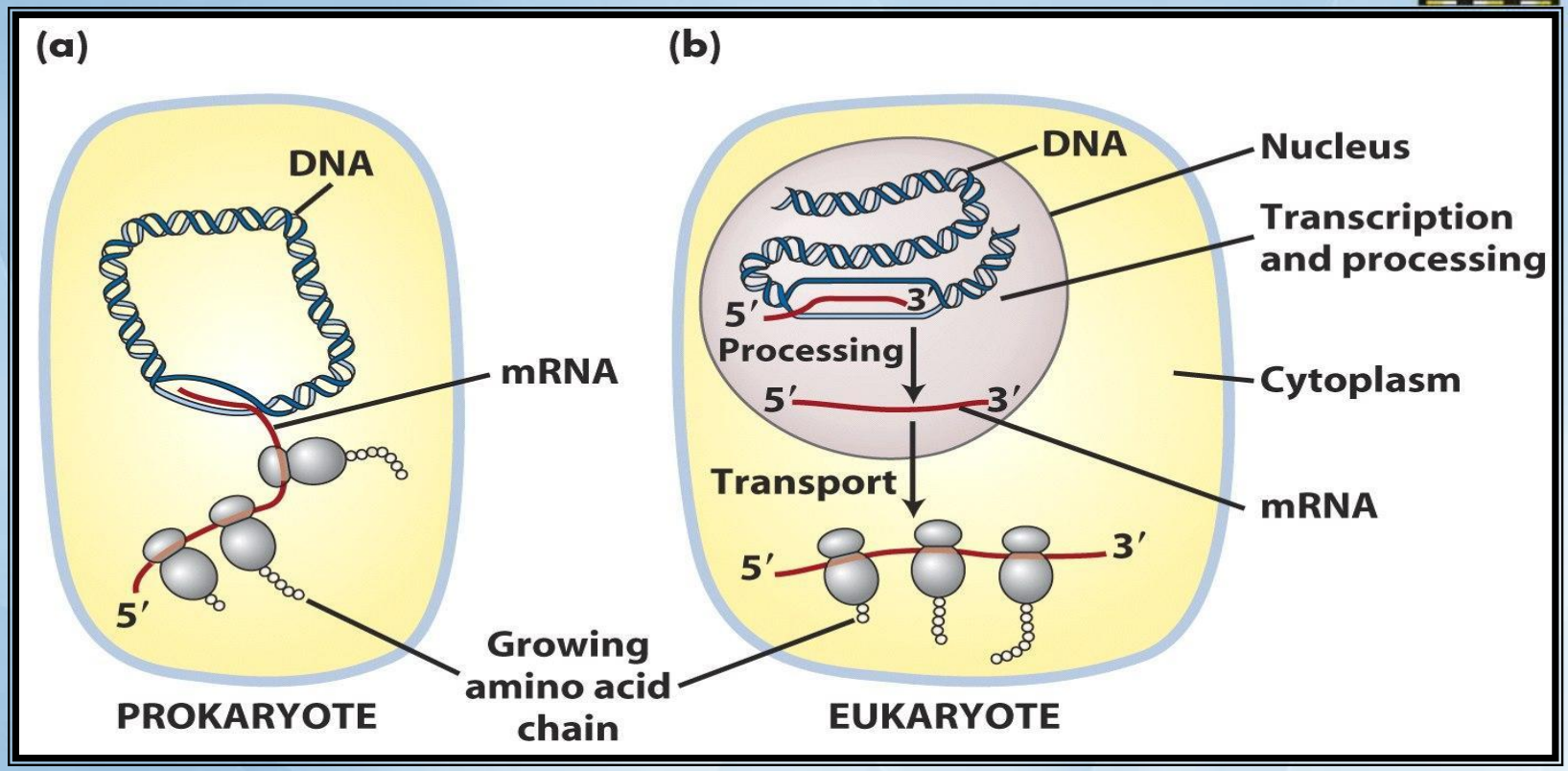
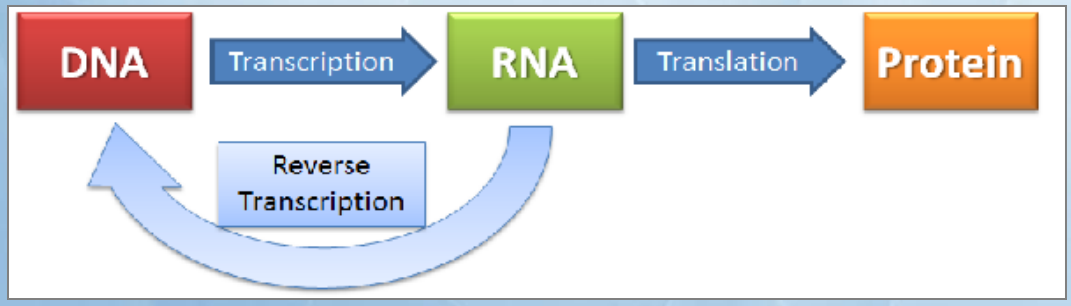

(a)
DNA
mRNA
5′
Growing amino acid chain
PROKARYOTE
(b)
DNA
Nucleus
Transcription and processing
5′
3′
Processing
5′
3′
Cytoplasm
Transport
mRNA
5′
3′
EUKARYOTE


DNA
Transcription
RNA
Translation
Protein
Reverse Transcription

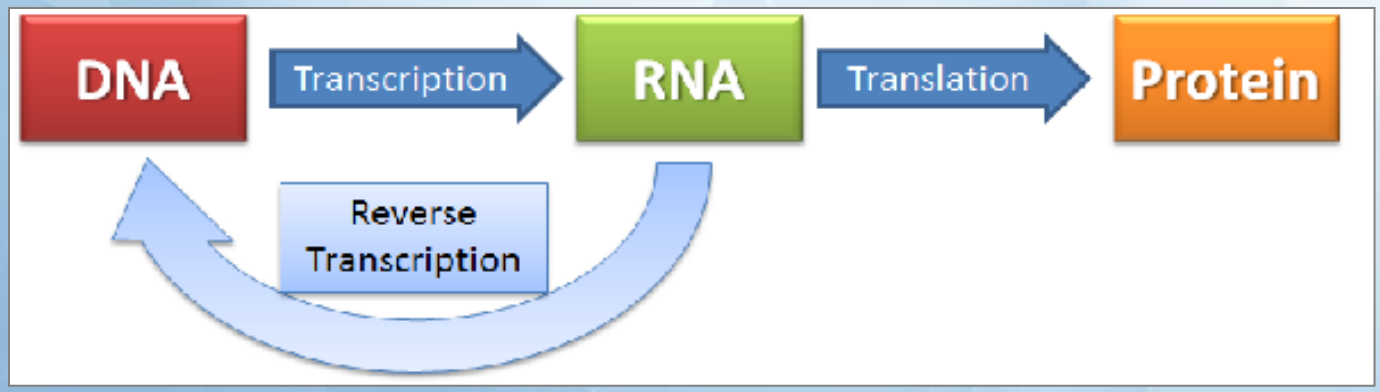
Proteins

- Make up about 15% of the cell
- Have many functions in the cell
  - Enzymes
  - Structural
  - Transport
  - Motor
  - Storage
  - Signaling
  - Receptors
  - Gene regulation
  - Special functions

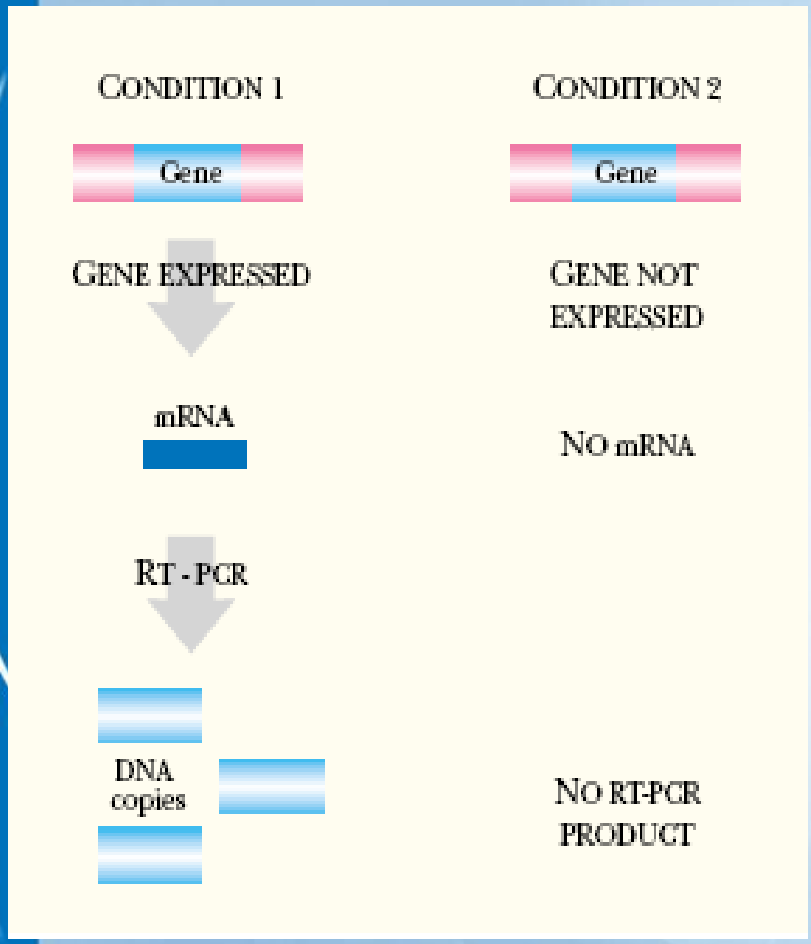
Expression analysis

Protein **mRNA**

Real-Time PCR
Micrroarray

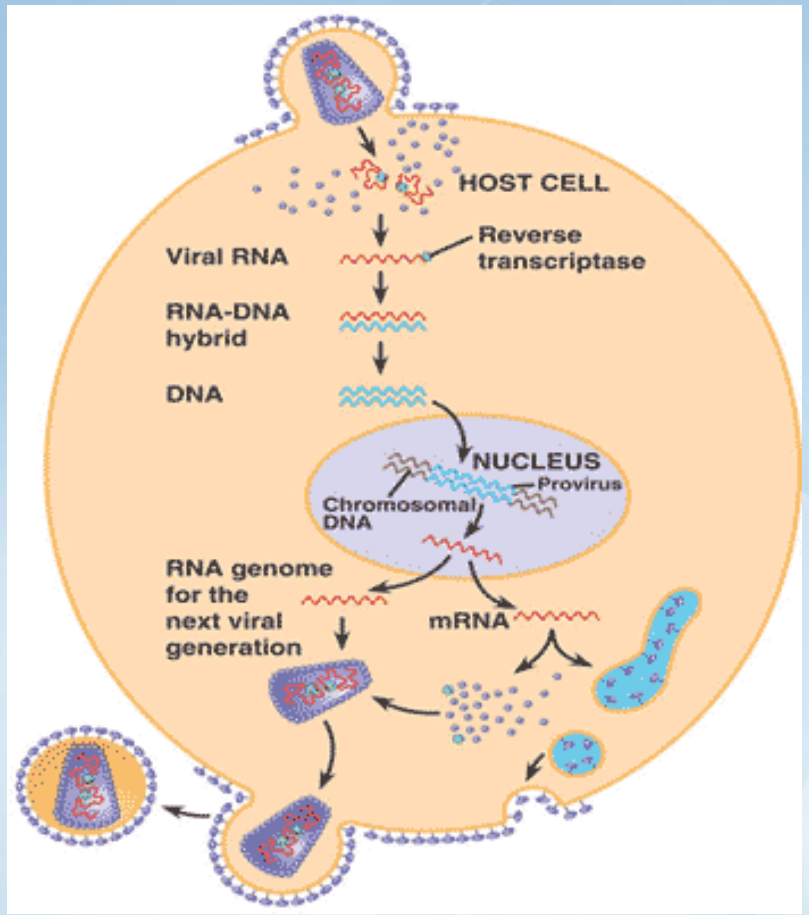


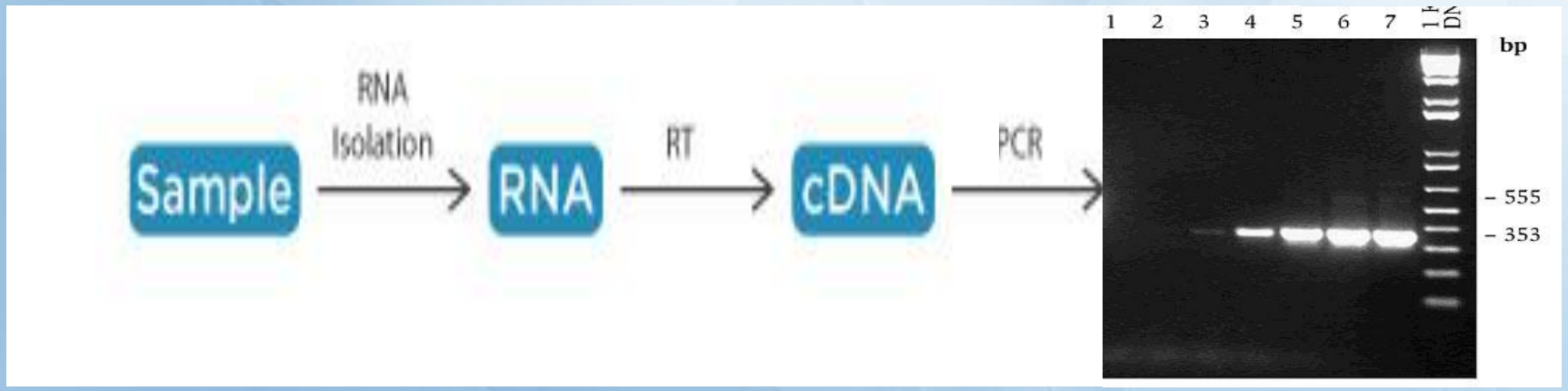
Why RT-PCR?



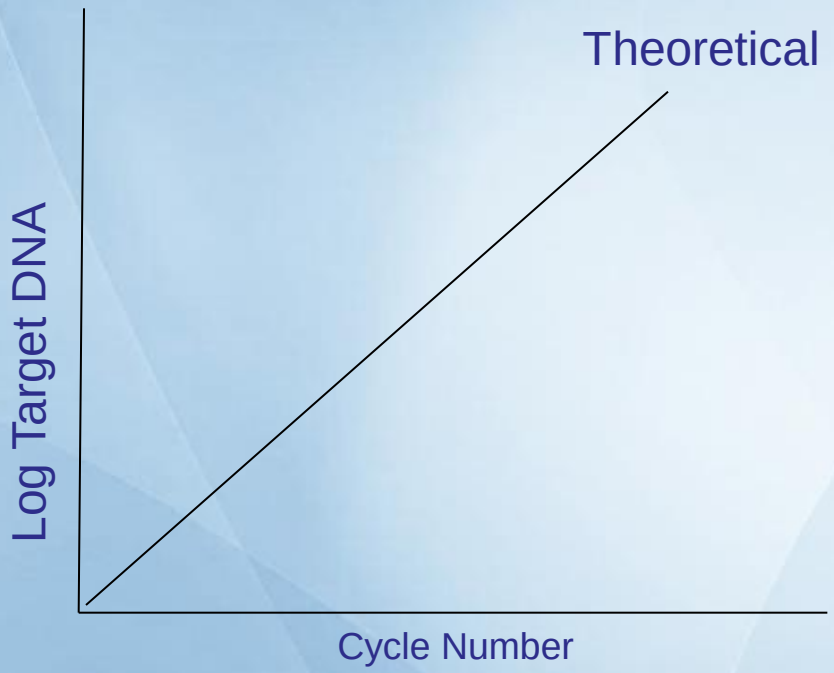
- **Gene expression analysis**
  - Cancer research
  - Drug research
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number

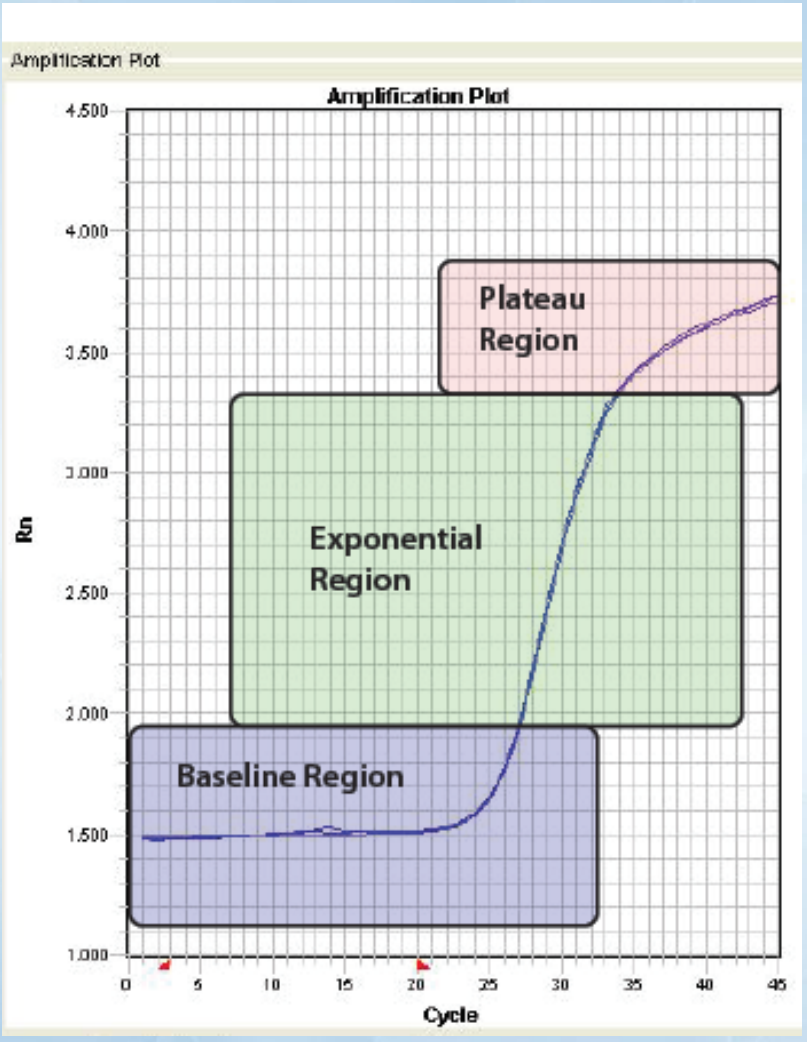
qRT-PCR is used to qualitatively detect gene expression


HOST CELL
Viral RNA
Reverse transcriptase
RNA-DNA hybrid
DNA
NUCLEUS
Provirus
Chromosomal DNA
RNA genome for the next viral generation
mRNA


Sample
RNA Isolation
RNA
RT
cDNA
PCR
1 2 3 4 5 6 7 L DN
bp
555
353

How does PCR work?

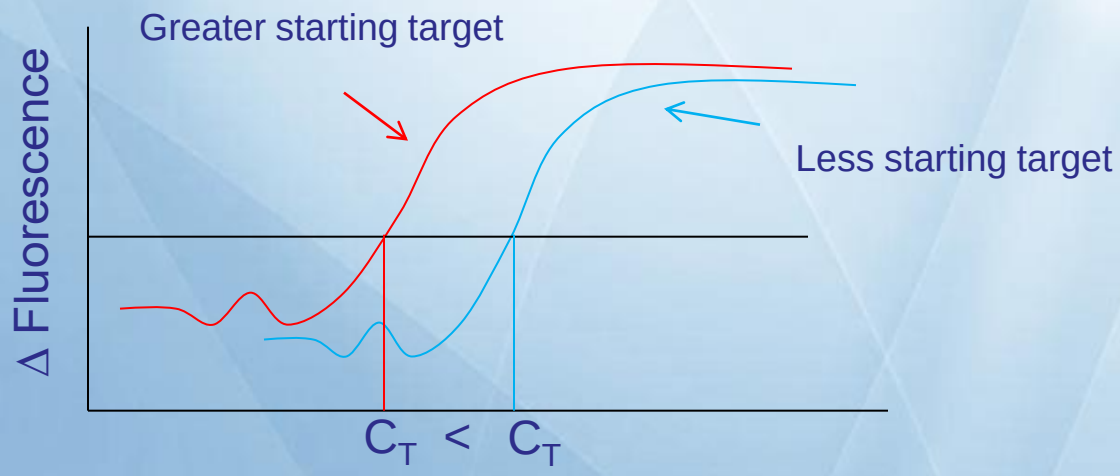




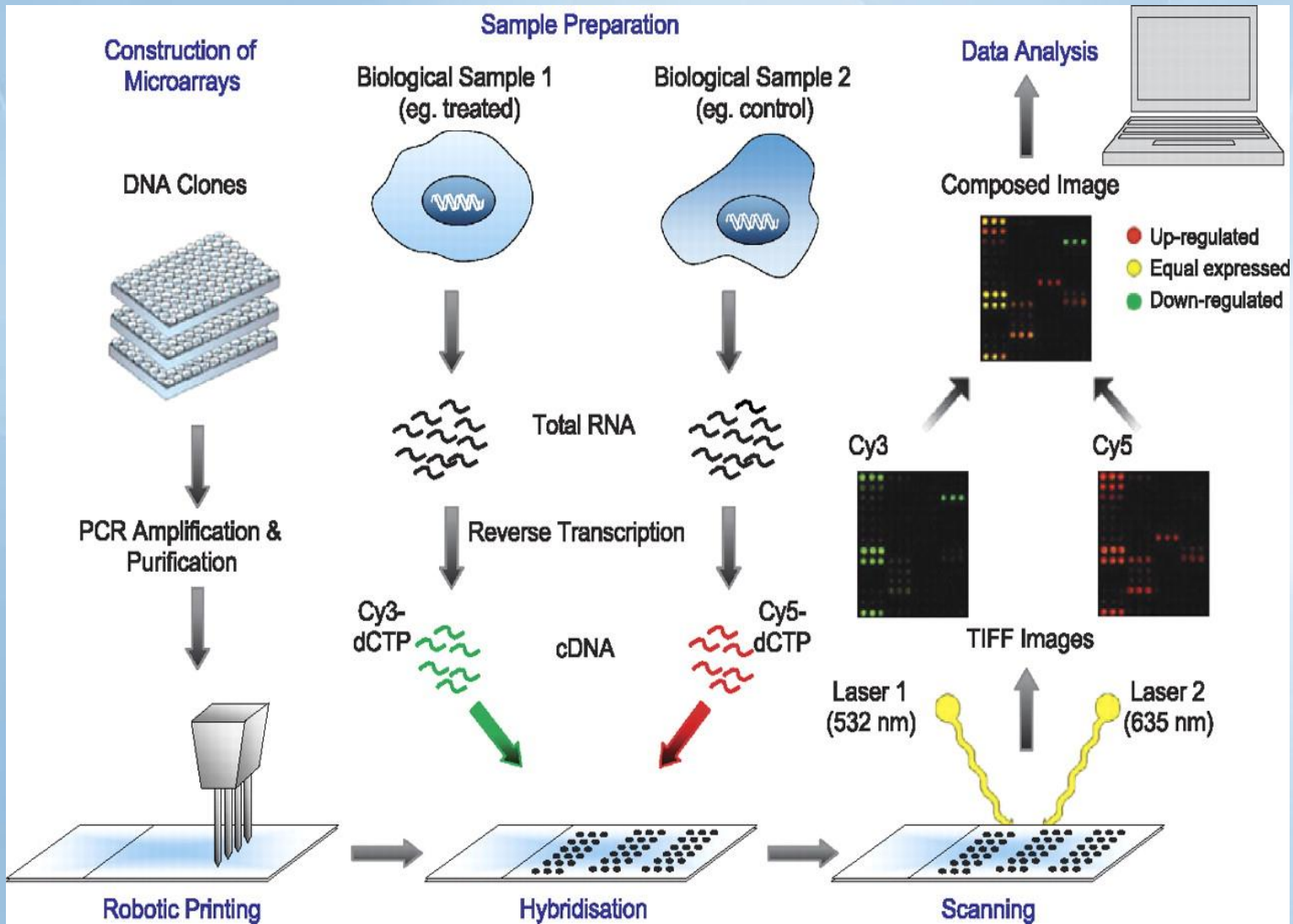
Real-Time PCR

- Real-Time PCR a specialized technique that allows a PCR reaction to be visualized “in real time” as the reaction progresses.

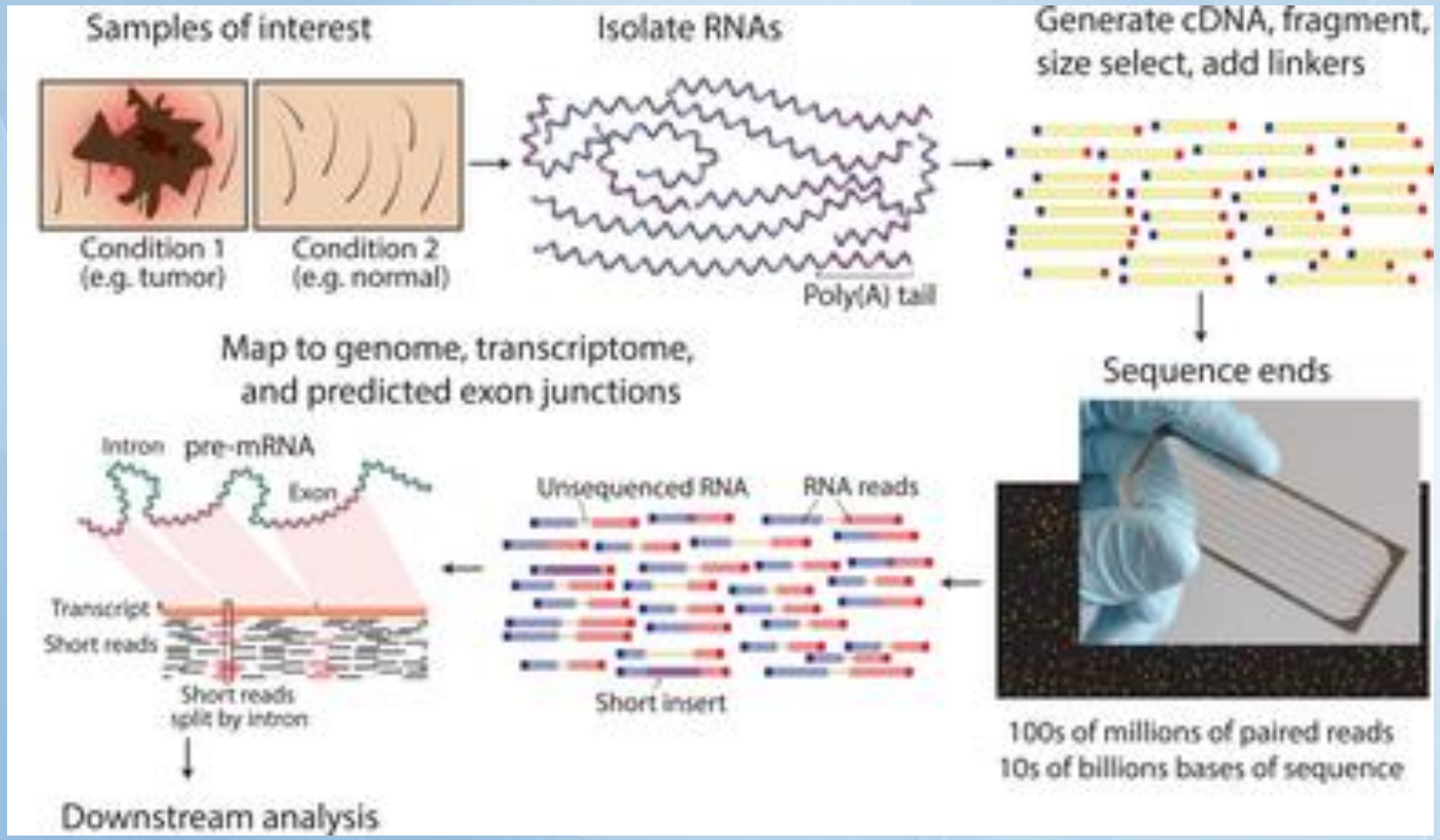
- <u>Quantitative PCR</u> relies on the principal that the quantity of target at the start of the reaction is proportional to amount of product produced during the exponential phase



Microarray

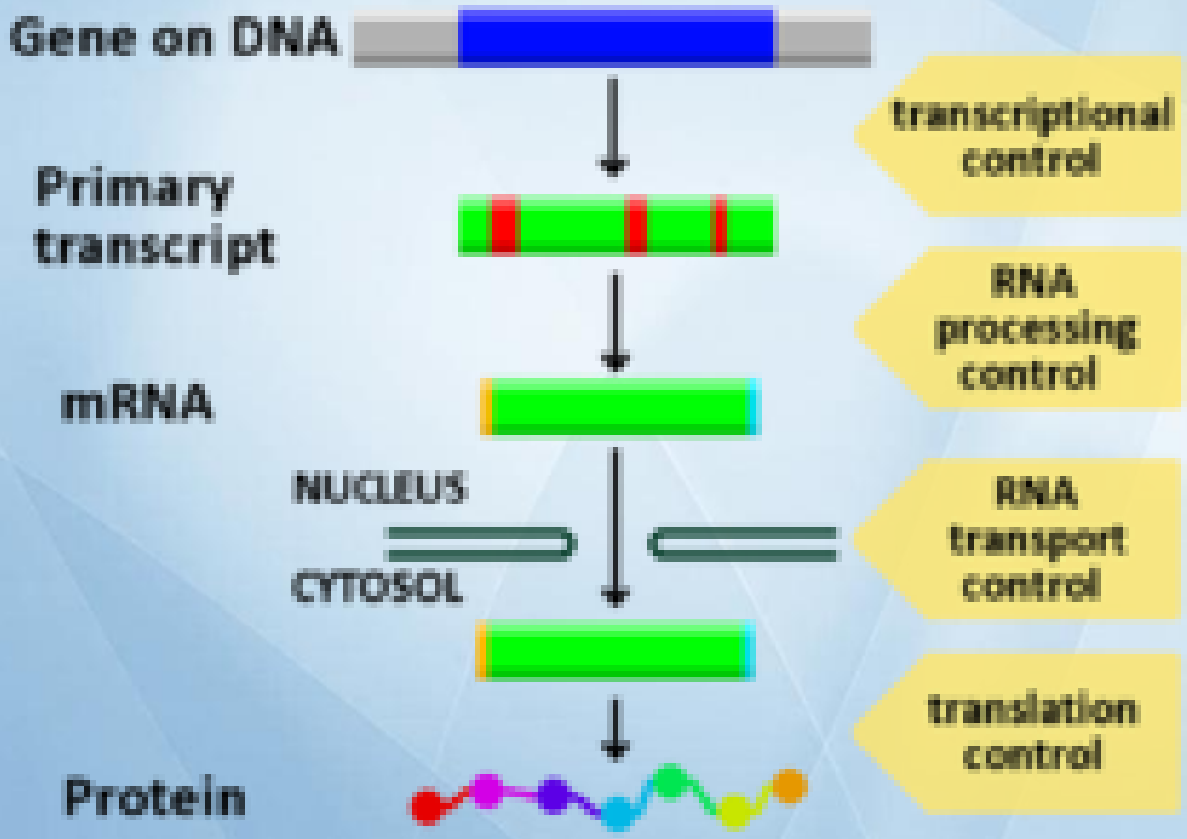

Construction of Microarrays
DNA Clones
PCR Amplification & Purification
Robotic Printing
Sample Preparation
Biological Sample 1 (eg. treated)
Biological Sample 2 (eg. control)
Total RNA
Reverse Transcription
Cy3-dCTP
Cy5-dCTP
cDNA
Hybridisation
Data Analysis
Composed Image
Up-regulated
Equal expressed
Down-regulated
Cy3
Cy5
TIFF Images
Laser 1 (532 nm)
Laser 2 (635 nm)
Scanning

RNA sequencing



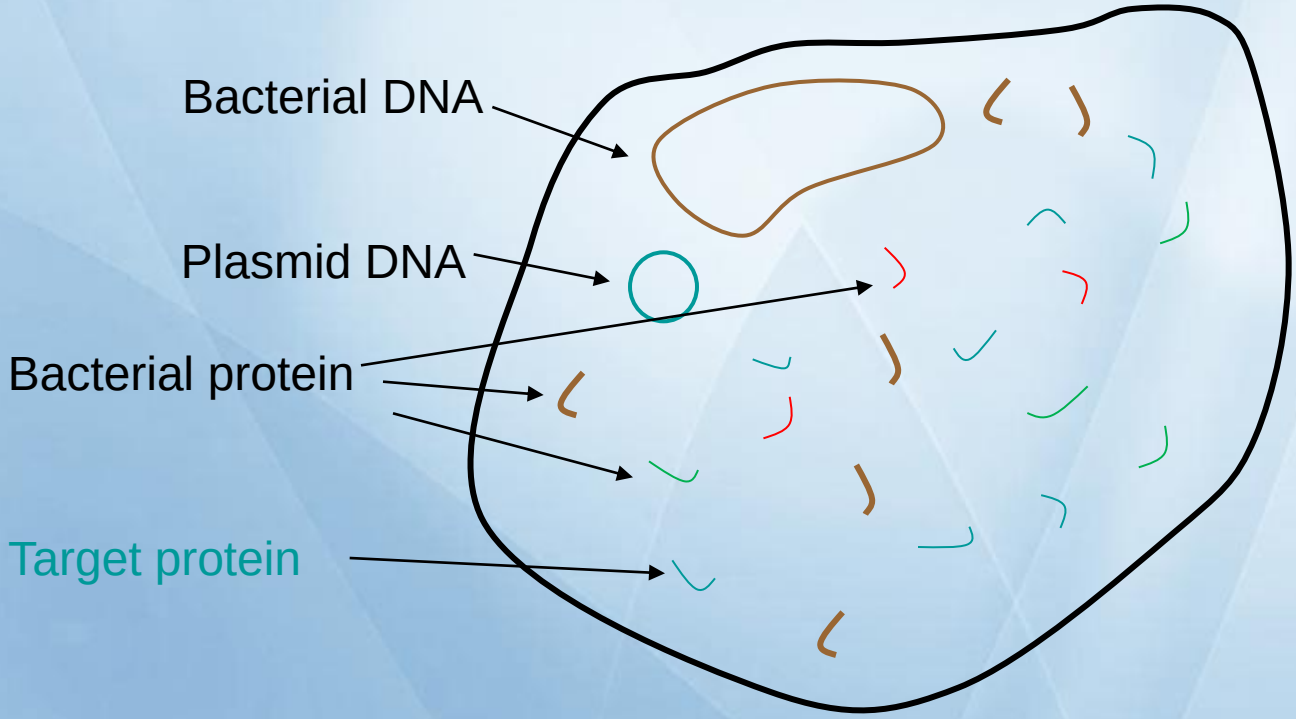
Expression analysis

Protein Level **mRNA**



Protein Quantification

- Most of proteins are colorless
- In most cases, a mixture of several proteins or many proteins



Quantification of Protein

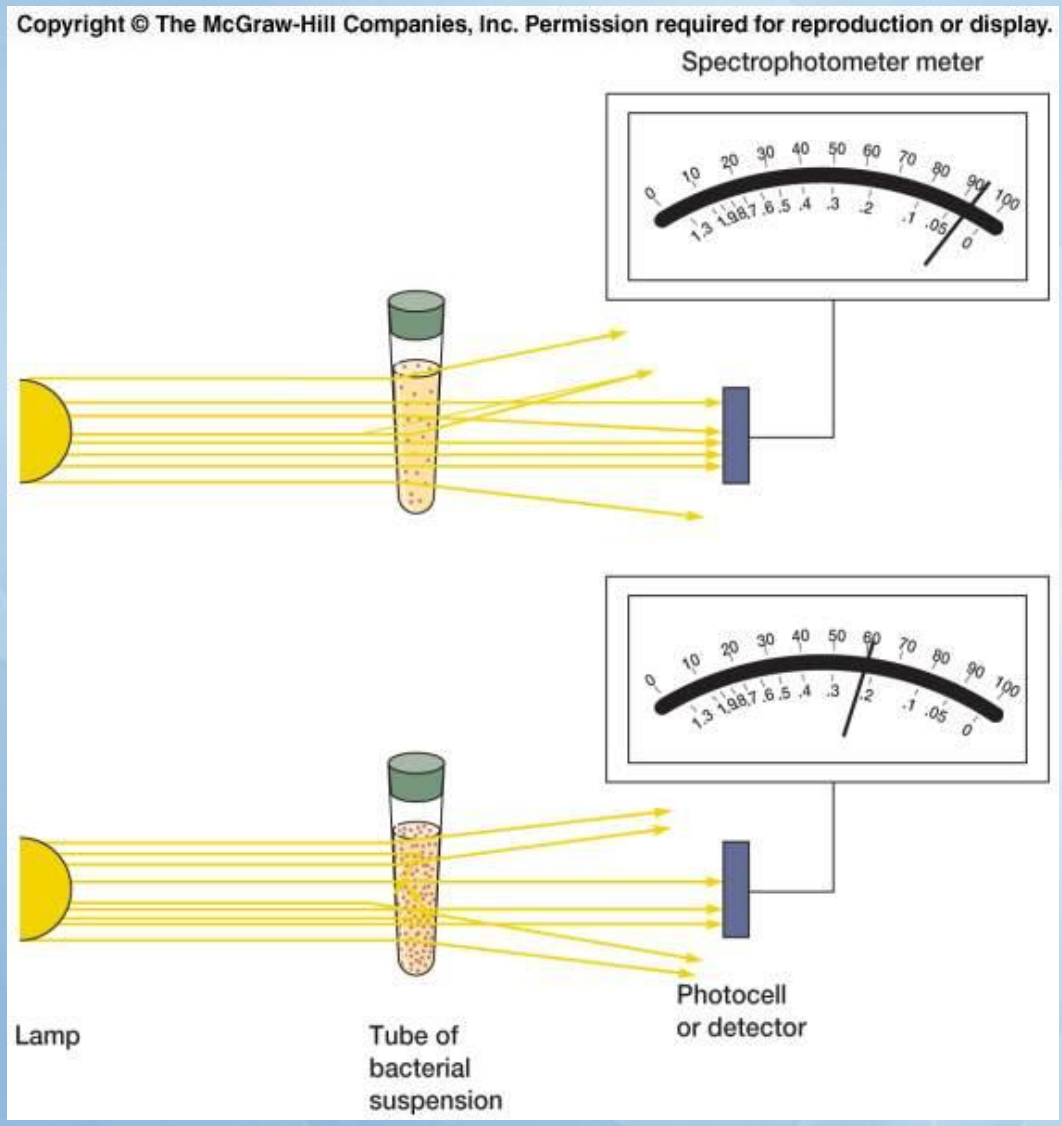
UV absorption

Colorimetric methods

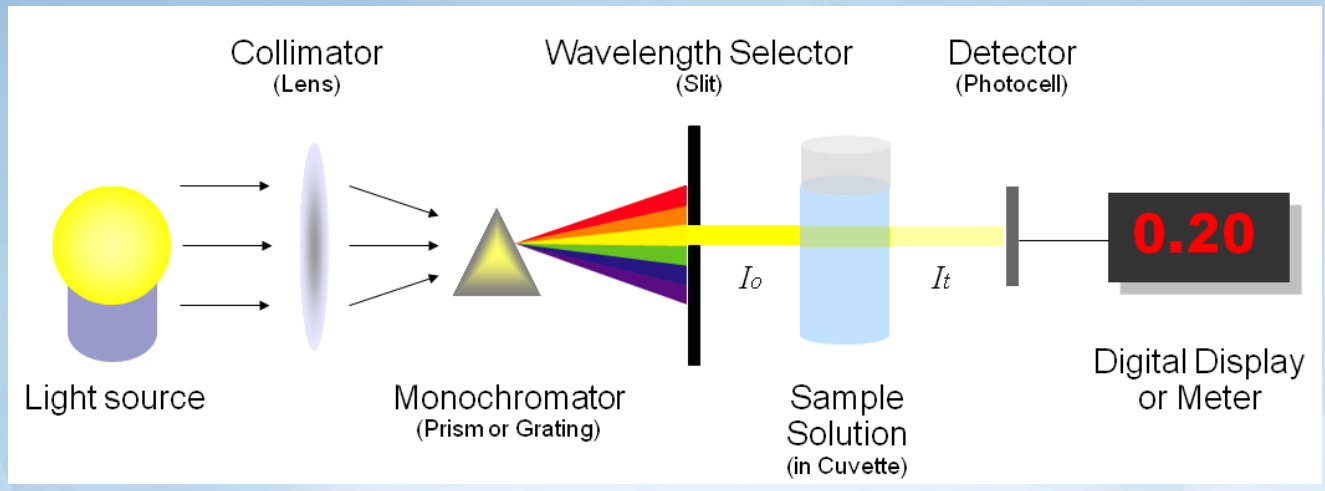
- Biuret
- Lowry
- Bradford

Other Methods

Absorbance


Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.
Spectrophotometer meter
0 10 20 30 40 50 60 70 80 90 100
1.3 1.9 .8 .7 .6 .5 .4 .3 .2 .1 .05 0
Lamp
Tube of bacterial suspension
Photocell or detector

UV Absorption Method



- The amino acids tryptophan, tyrosine and phenylalanine absorb light in the UV wavelength (280 nm)

- Since the absorption is proportional to concentration, this is a useful way to quantitate protein concentration (for proteins containing Trp)

Advantages:

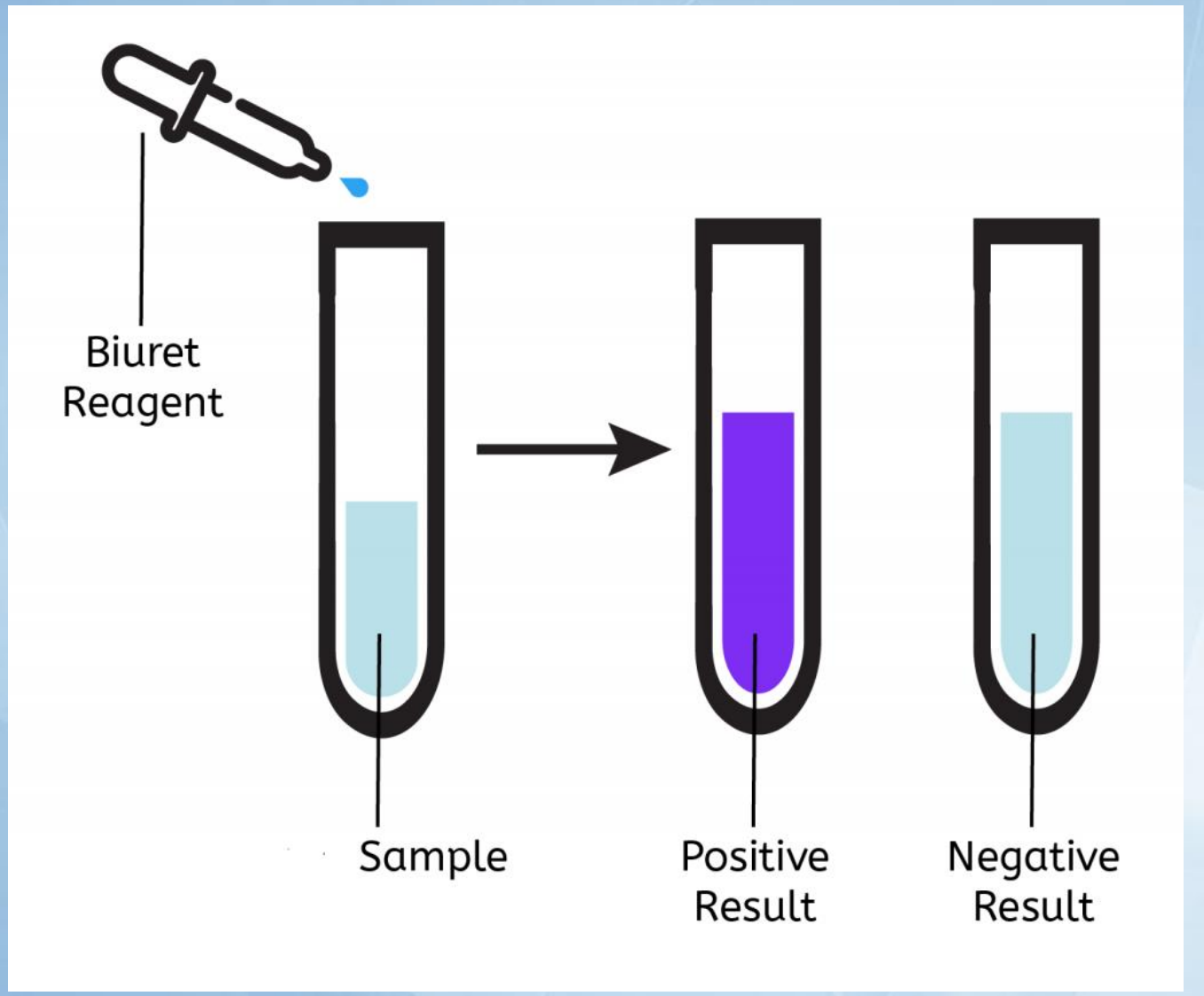
1. Rapid
2. Non interference from ammonium sulfate.
3. Non destructive.

Disadvantages

- If some proteins do not contain these amino acids, it will not absorb UV light.
- Turbidity (cloudiness in solution) is a problem.
- Nucleic acids (DNA, RNA) contaminant and phenolic acids will also absorb UV light.

Colorimetric Methods

- we can modify the protein sample with appropriate reagents so as to produce a color reaction and measure protein concentration using a spectrophotometer.

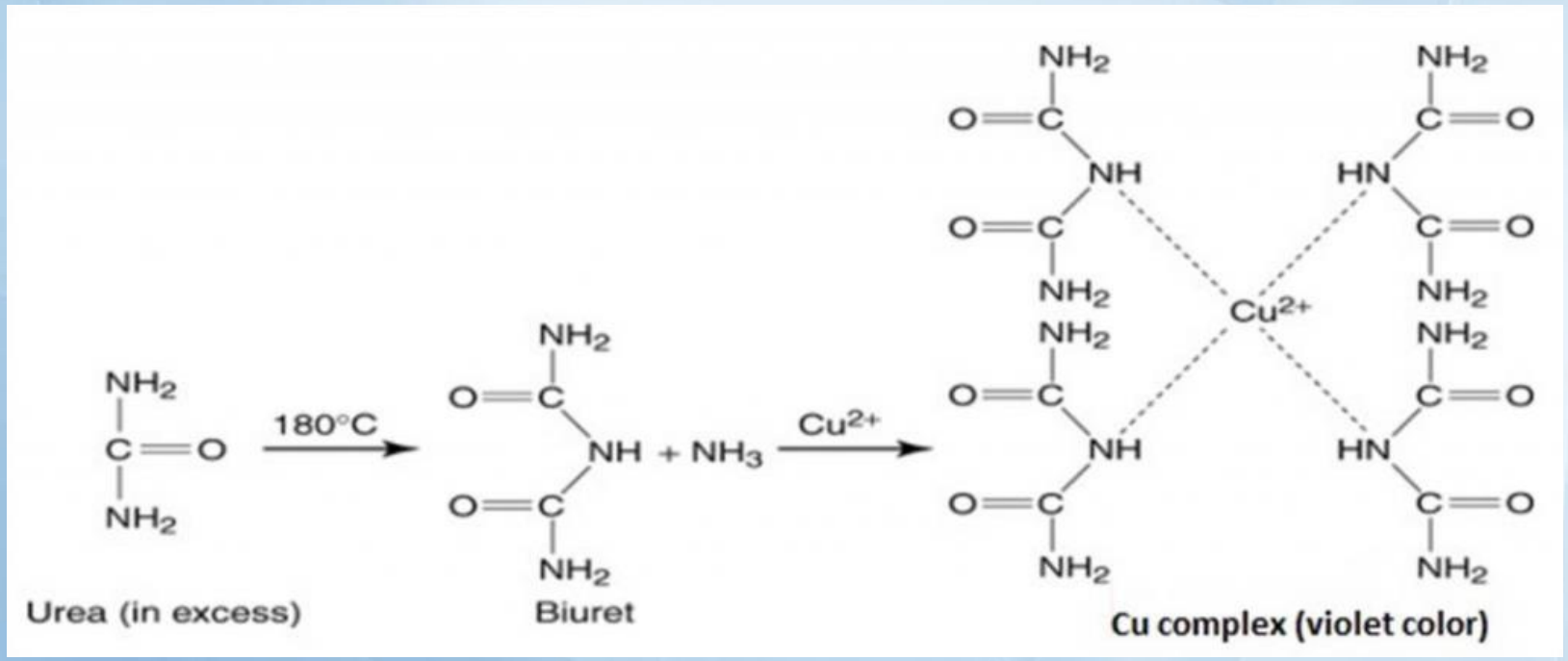

Biuret
Reagent
Sample
Positive
Result
Negative
Result

Advantages of Colorimetric Methods

1. Cheap
2. Not contaminating absorbance from nucleic acids!

- Biuret
- Lowry
- Bradford

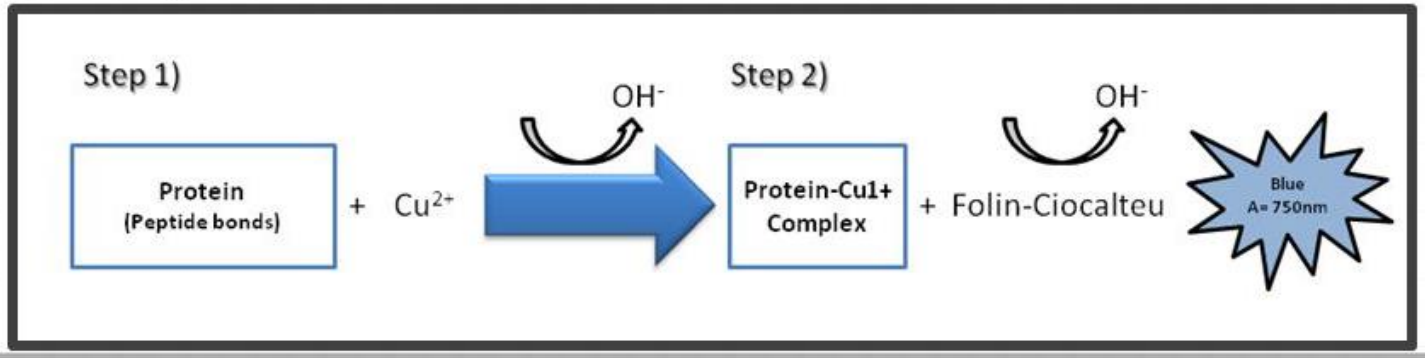
Biuret



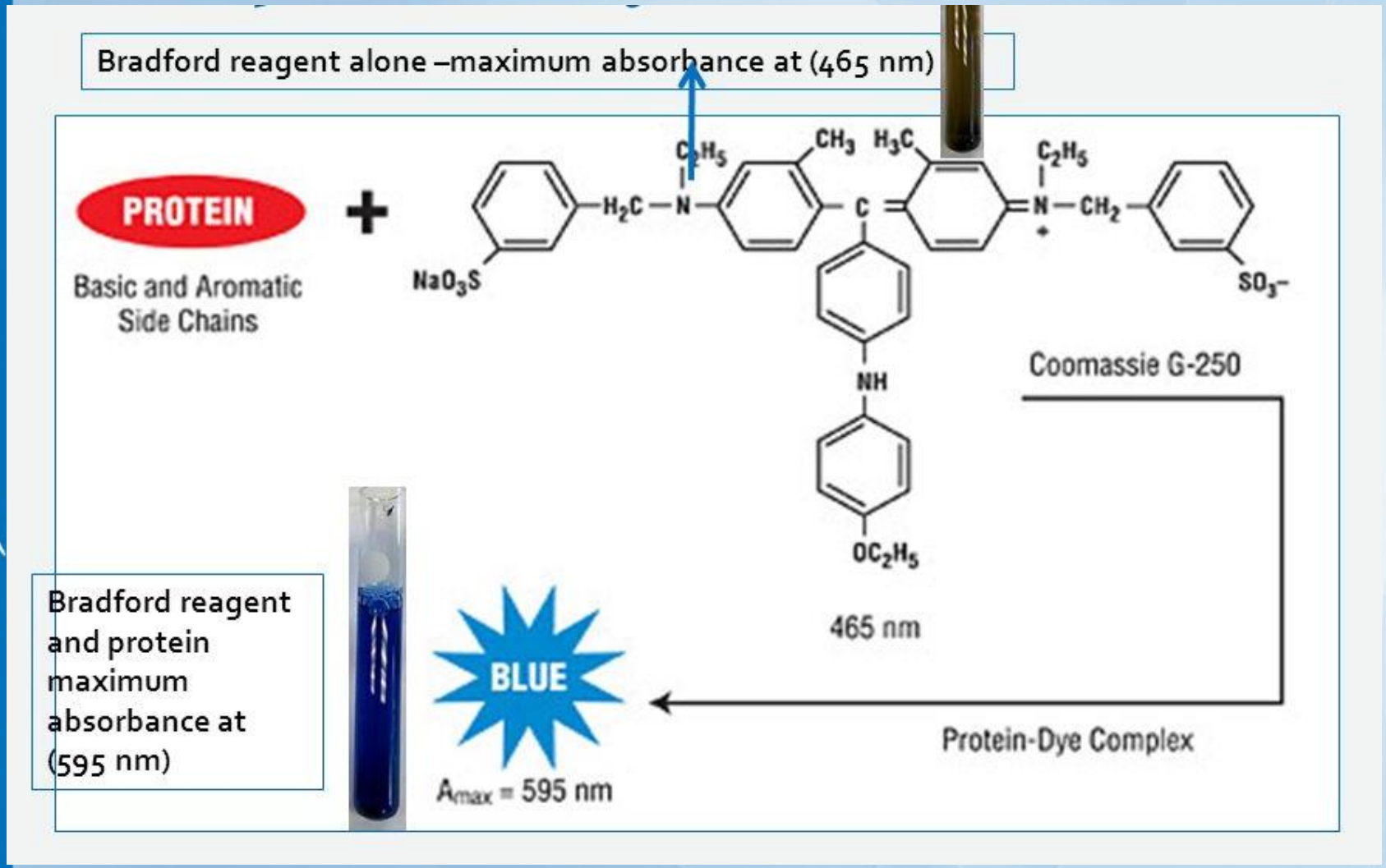
Lowry

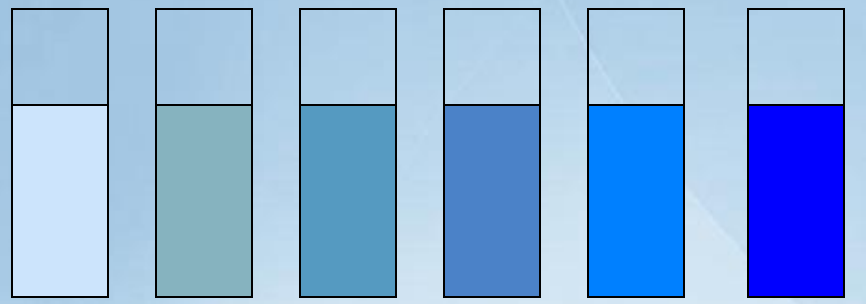
- **Is based on two chemical reactions:**

- **The first reaction** is the reduction of copper ions under alkaline conditions, which forms a complex with peptide bonds .
- **The second** is the reduction of Folin-Ciocalteu reagent by the copper-peptide bond complex, which subsequently causes a color change of the solution into blue with an absorption in the range of 650 to 750 nm detectable with a spectrophotometer.



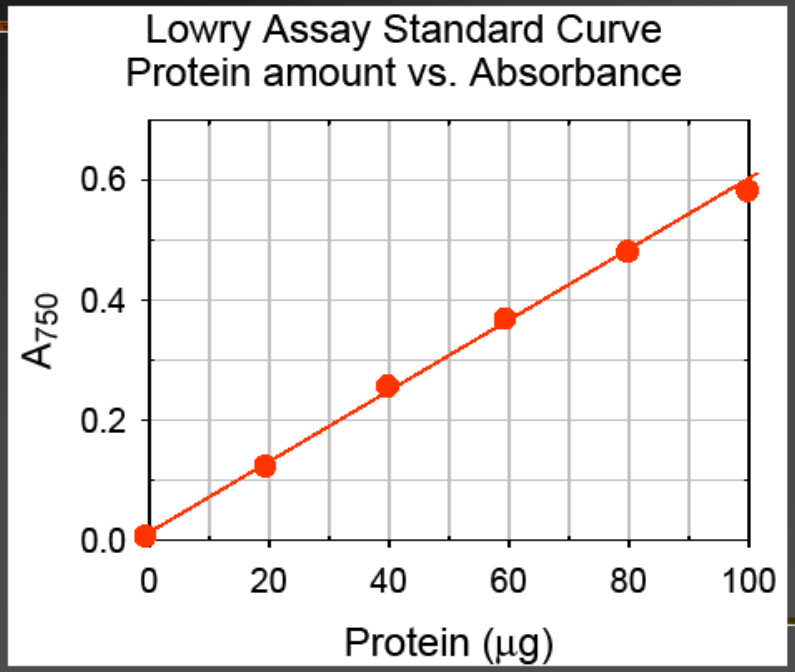
Bradford


Bradford reagent alone –maximum absorbance at (465 nm)
PROTEIN
Basic and Aromatic Side Chains
Coomassie G-250
465 nm
BLUE
A_{max} = 595 nm
Protein-Dye Complex
Bradford reagent and protein maximum absorbance at (595 nm)



2.5 5 10 15 20 25 µg/mL

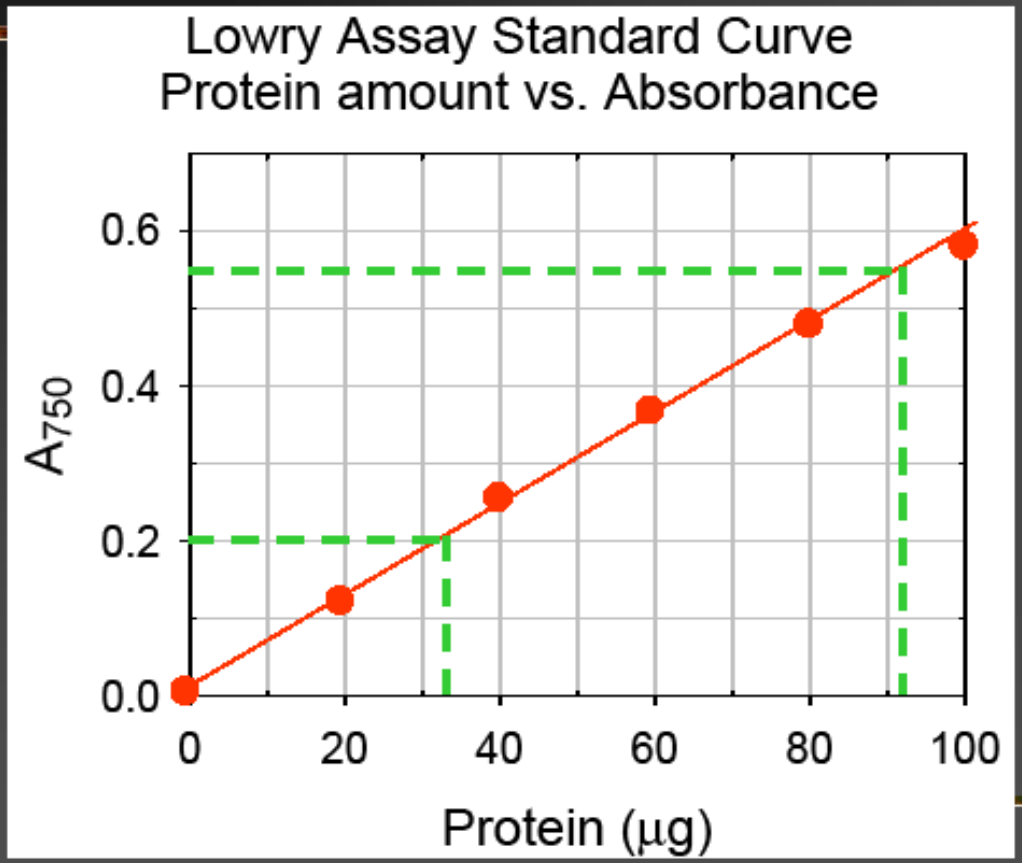
Standard Curve



Protein standards:

Protein (µg)	A_{750}
0	0.000
20	0.120
40	0.254
60	0.372
80	0.480
100	0.601

Using Standard Curve



Protein unknowns:

Protein (µg)	A_{750}
33	0.200
92	0.550

Protein Quantification Methods

Assay method	Useful range	Comments
BCA method (Biuret)	0.5ug/ml to 1.5mg.ml	•Samples must be read within 10 mins •Not compatible with reducing agents
Bradford assay (dye binding)	1ug/ml to 1.5 mg/ml	•Protein precipitates over time •High protein to protein signal variability •Not compatible with detergents
Lowry assay	1ug/ml to 1.5mg.ml	•Lengthy, multi-step procedure •Not compatible with detergents, carbohydrates or reducing agents
Absorbance at 280nm	50ng/ml to 2mg/ml	•High protein to protein signal variability •Detection influenced by nucleic acids and other UV absorbing contaminants

Kjeldahl method

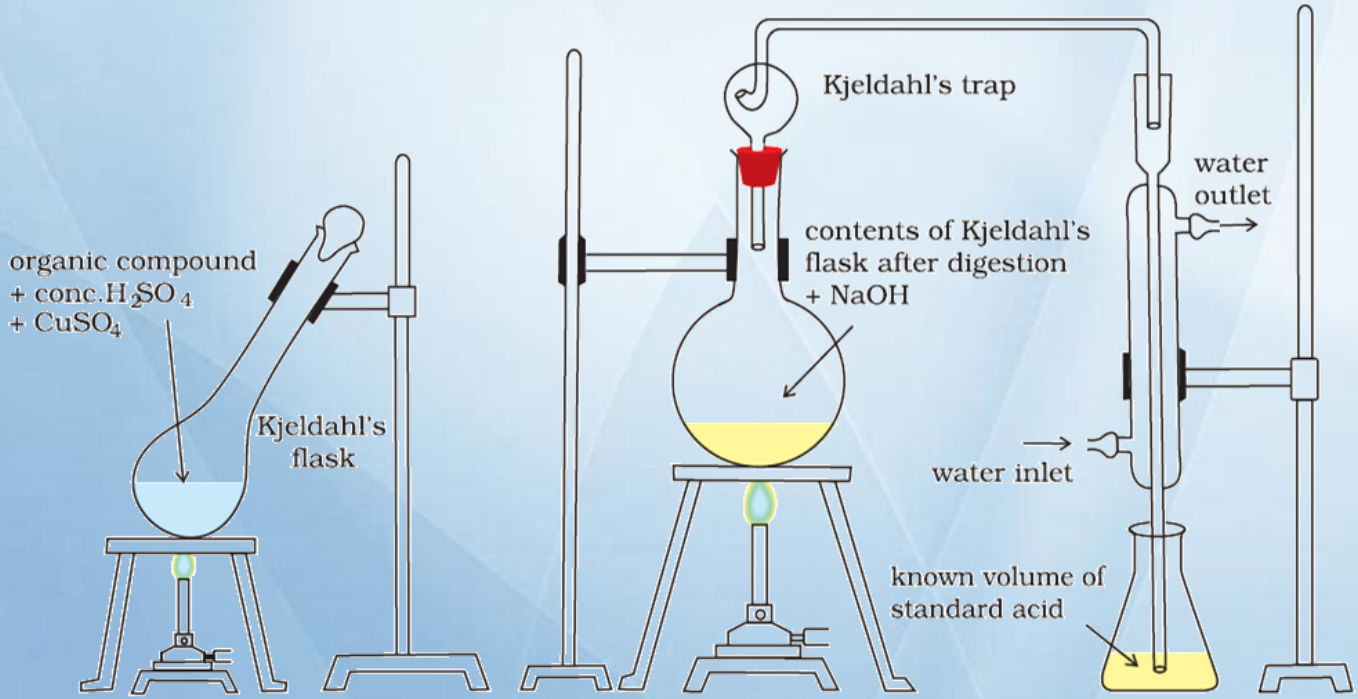
PRINCIPLE:

The method consists of three basic steps:

1) Digestion of the sample in sulfuric acid with a catalyst, which results in conversion of nitrogen to ammonia;

2) Distillation of the ammonia into a trapping solution; and

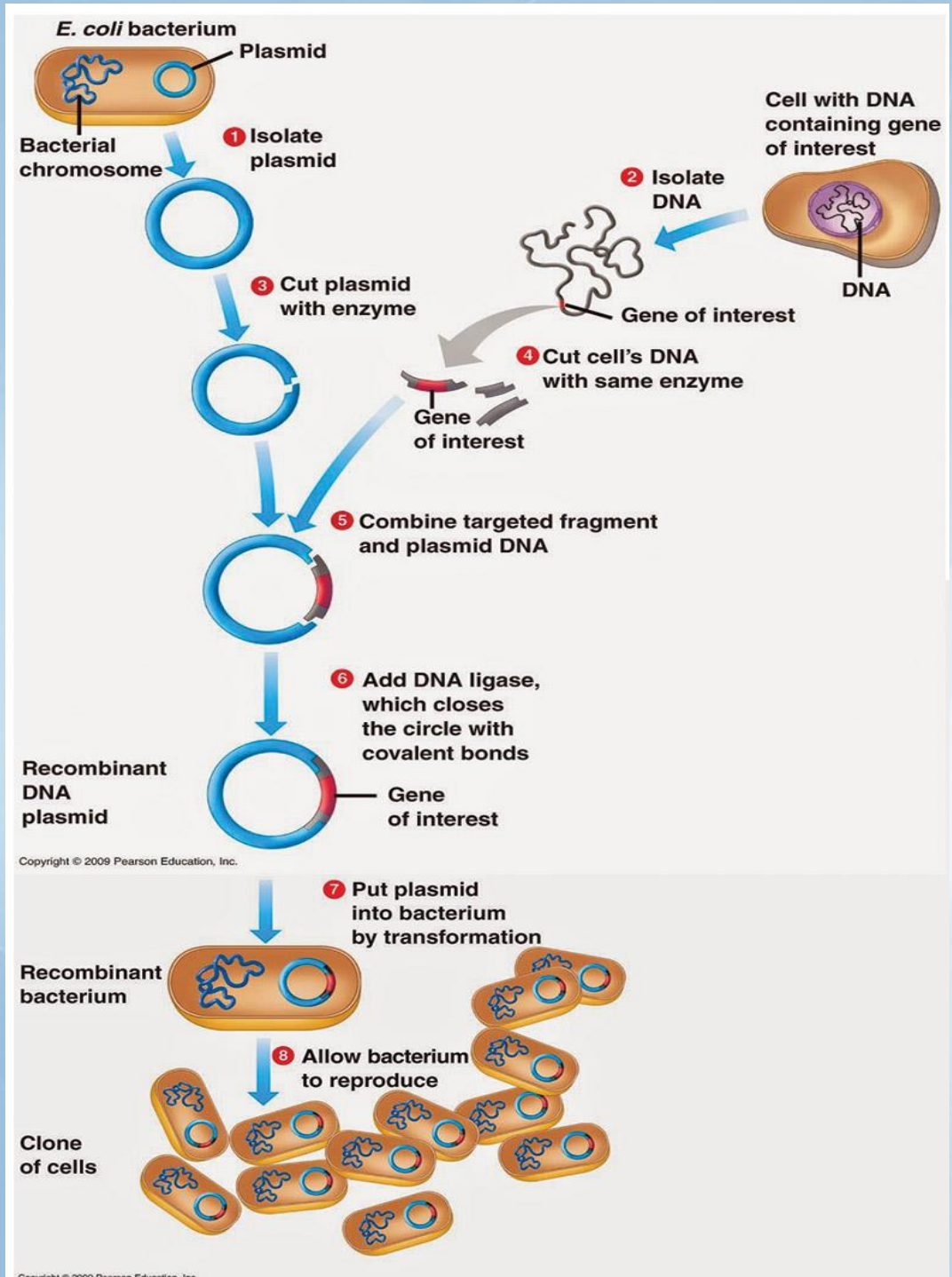
3) Quantification of the ammonia by titration with a standard solution



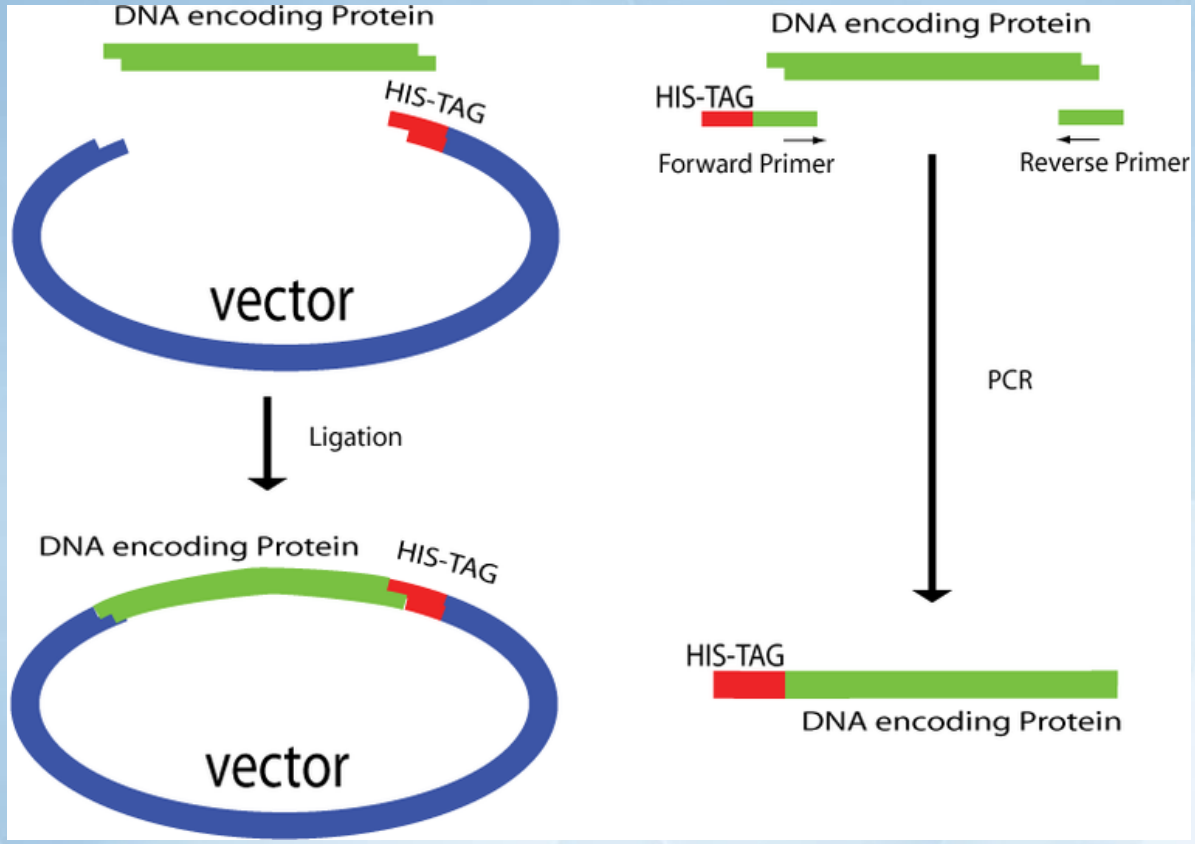
Why purify a protein?

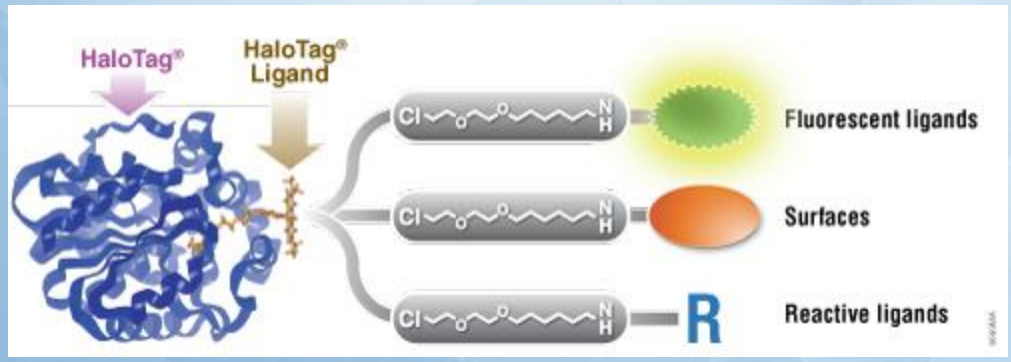
- To study its function, Activity
- For industrial or therapeutic applications
- Study protein regulation and protein interactions
- Produce Antibodies
- Perform structural analysis by X-Ray and Crystallography

Gene cloning



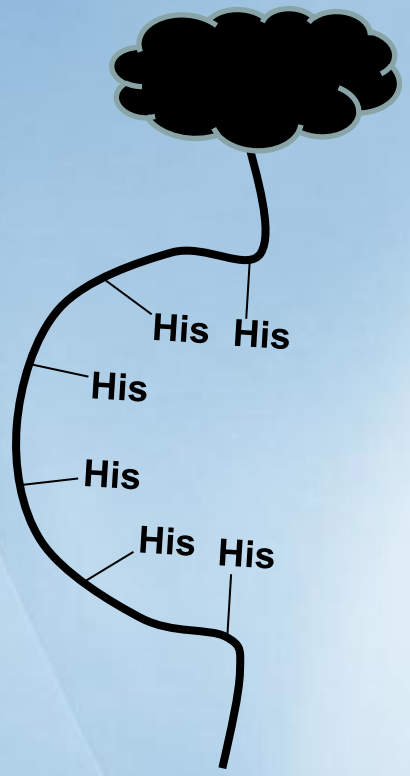
Protein tags

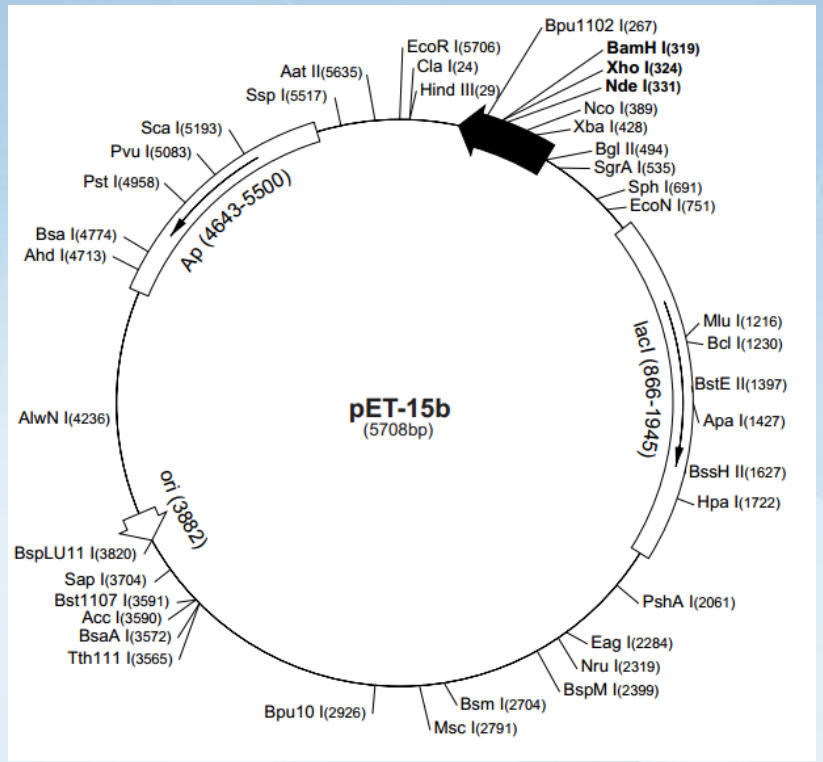


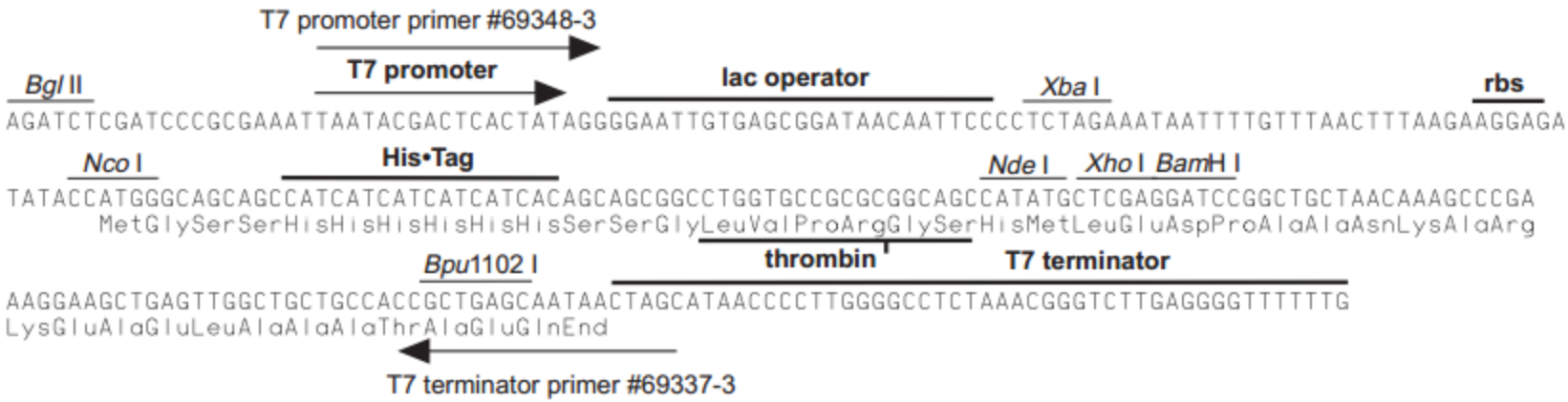


Examples of tags and ligands

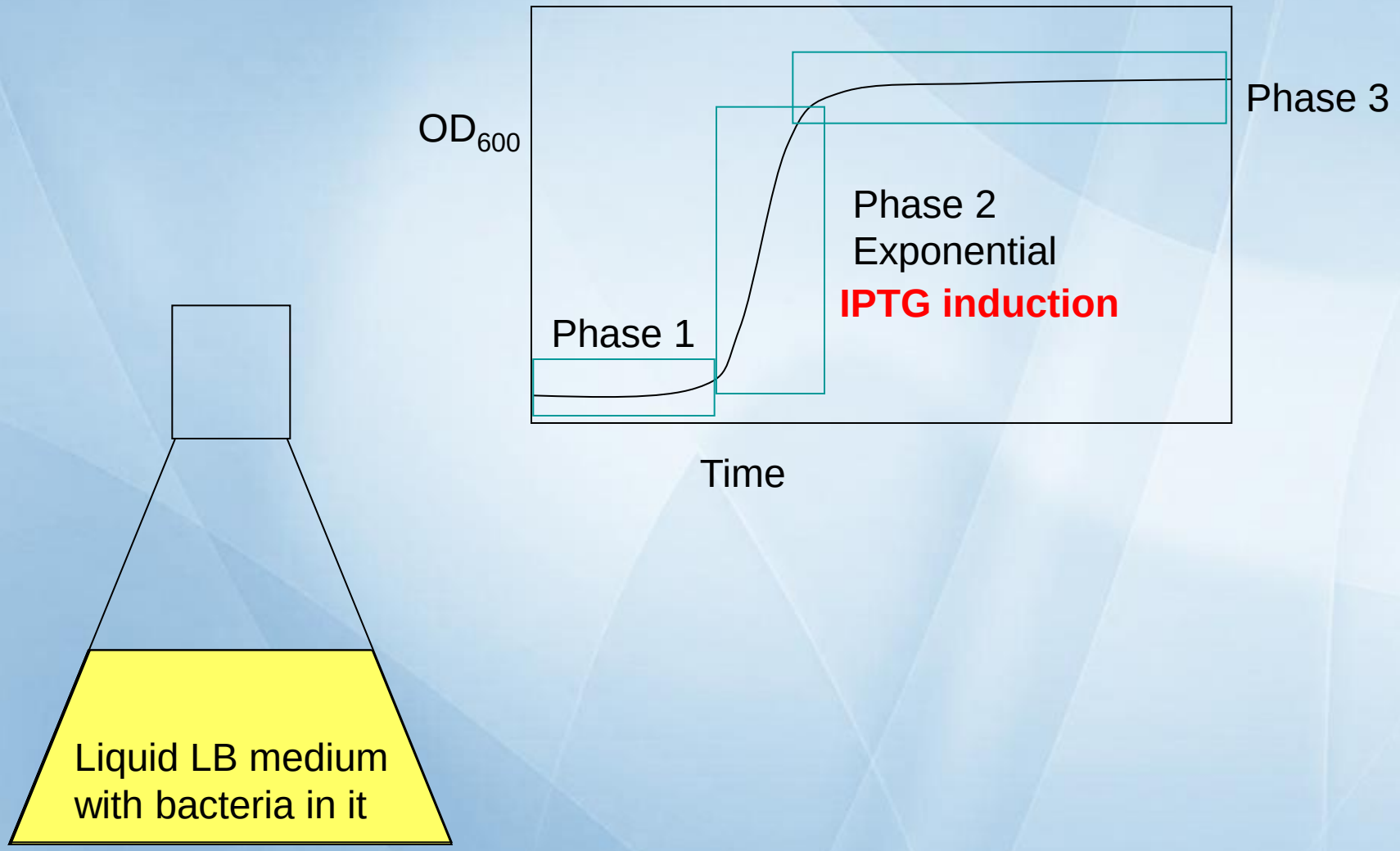
- His-tag
- FLAG™ peptide
- *Strep*-tag
- GST tag
- Maltose binding protein fusion
- Calmodulin binding protein fusion
- Transition metal ion
- Monoclonal antibody
- Biotin
- Glutathione
- Amylose
- Ca^{2+}


His
His
His
His
His
His

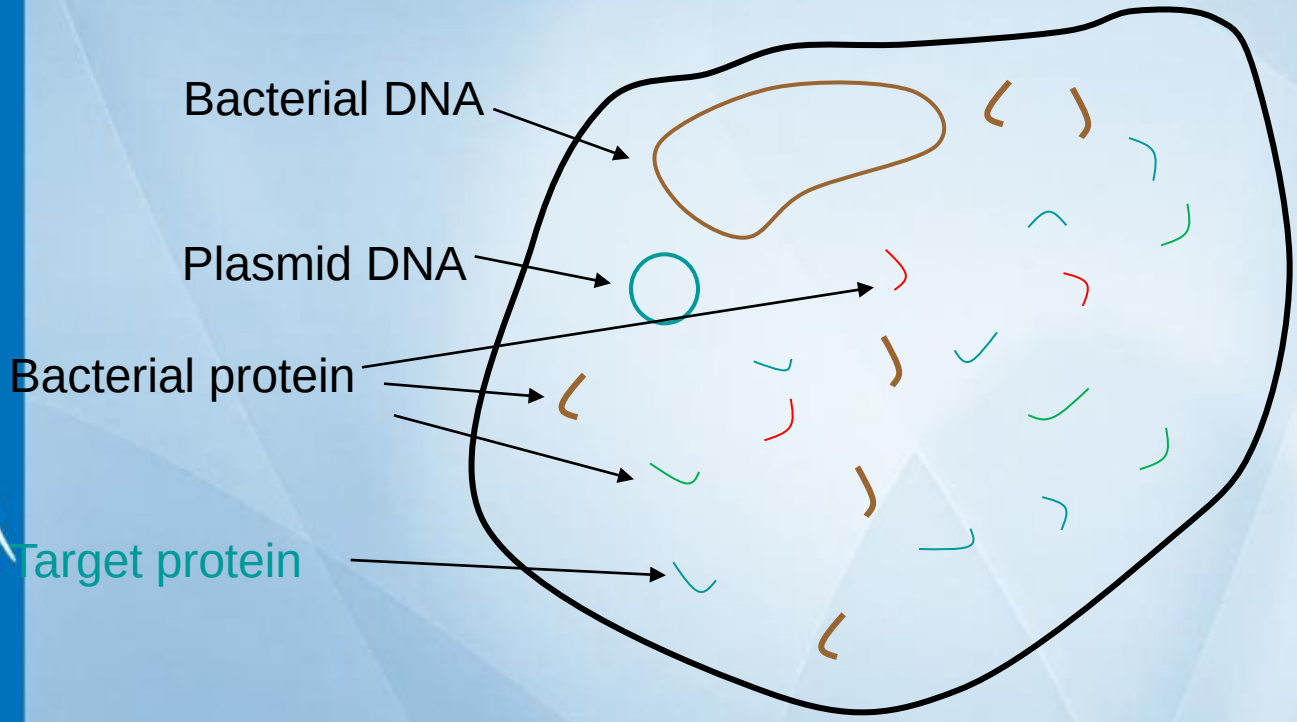

pET-15b
(5708bp)
Bpu1102 I(267)
BamH I(319)
Xho I(324)
Nde I(331)
EcoR I(5706)
Cla I(24)
Hind III(29)
Nco I(389)
Xba I(428)
Bgl II(494)
SgrA I(535)
Sph I(691)
EcoN I(751)
Aat II(5635)
Ssp I(5517)
Sca I(5193)
Pvu I(5083)
Pst I(4958)
Bsa I(4774)
Ahd I(4713)
Ap (4643-5500)
AlwN I(4236)
ori (3882)
BspLU11 I(3820)
Sap I(3704)
Bst1107 I(3591)
Acc I(3590)
BsaA I(3572)
Tth111 I(3565)
Bpu10 I(2926)
Bsm I(2704)
Msc I(2791)
BspM I(2399)
Nru I(2319)
Eag I(2284)
PshA I(2061)
Hpa I(1722)
BssH II(1627)
Apa I(1427)
BstE II(1397)
Bcl I(1230)
Mlu I(1216)
lacI (866-1945)

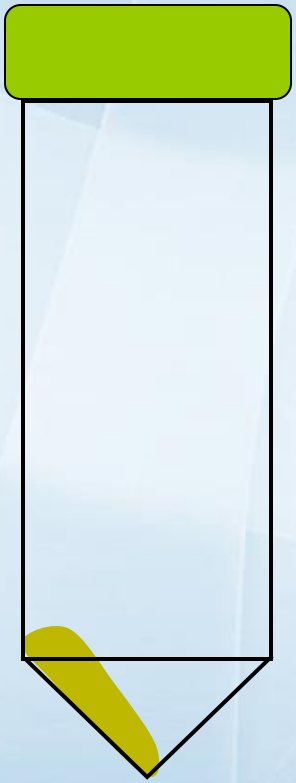

T7 promoter primer #69348-3
T7 promoter
lac operator
Bgl II
Xba I
rbs
AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA
Nco I
His•Tag
Nde I
Xho I
BamH I
TATACCATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCTCGAGGATCCGGCTGCTAACAAAGCCCGA
MetGlySerSerHisHisHisHisHisHisSerSerGlyLeuValProArgGlySerHisMetLeuGluAspProAlaAlaAsnLysAlaArg
thrombin
Bpu1102 I
T7 terminator
AAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
LysGluAlaGluLeuAlaAlaAlaThrAlaGluGlnEnd
T7 terminator primer #69337-3

Protein Expression



Bacterial Growth

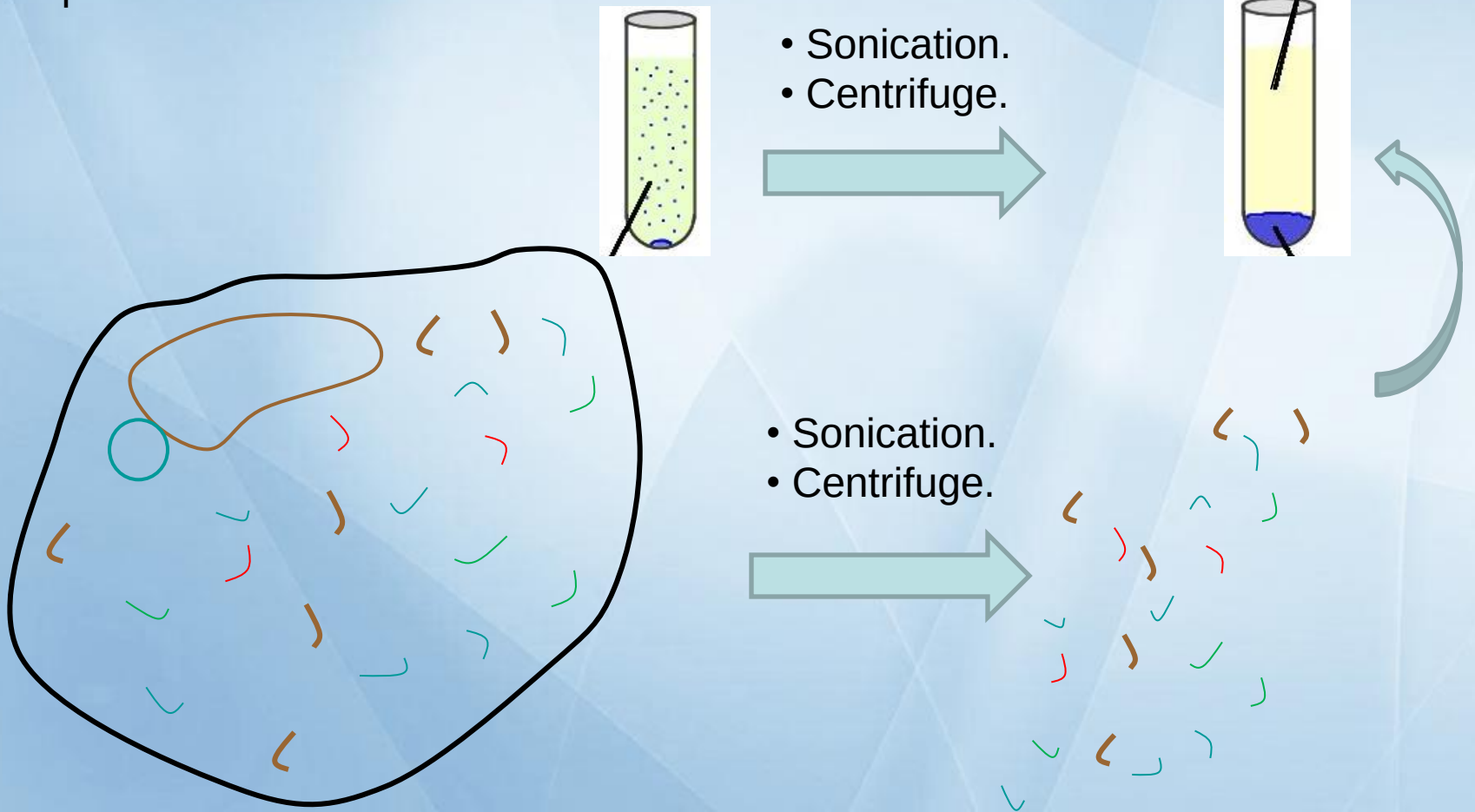




Lysis

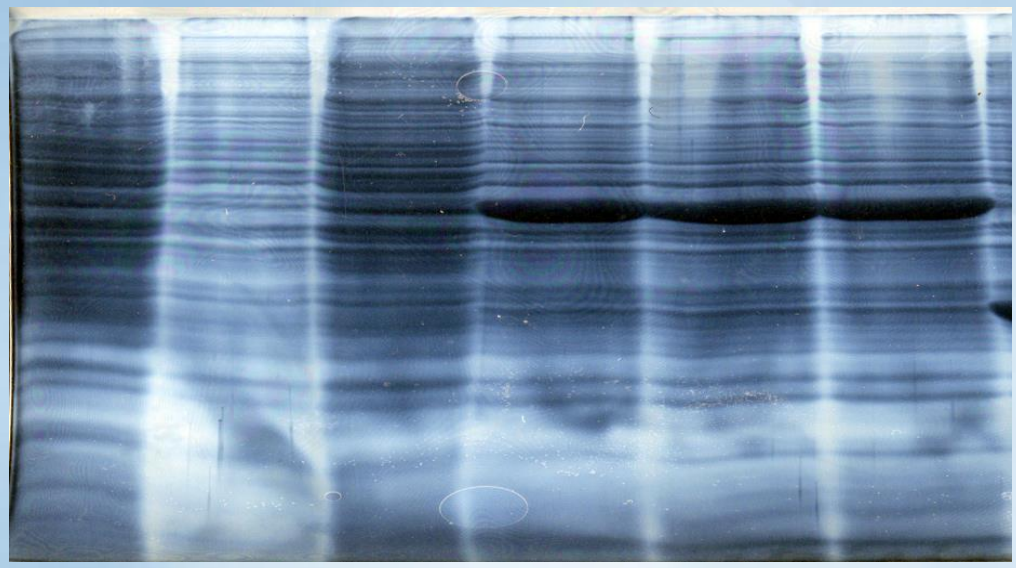
•Pellet is resuspended in the lysis buffer containing, and sonicated to further liberate the protein

• Spin down the denaturing lysis buffer, cell wall and debris will pellet at the bottom and our protein is in the soluble supernatant.



Expression of protein in *E. coli*

Uninduced Induced Samples



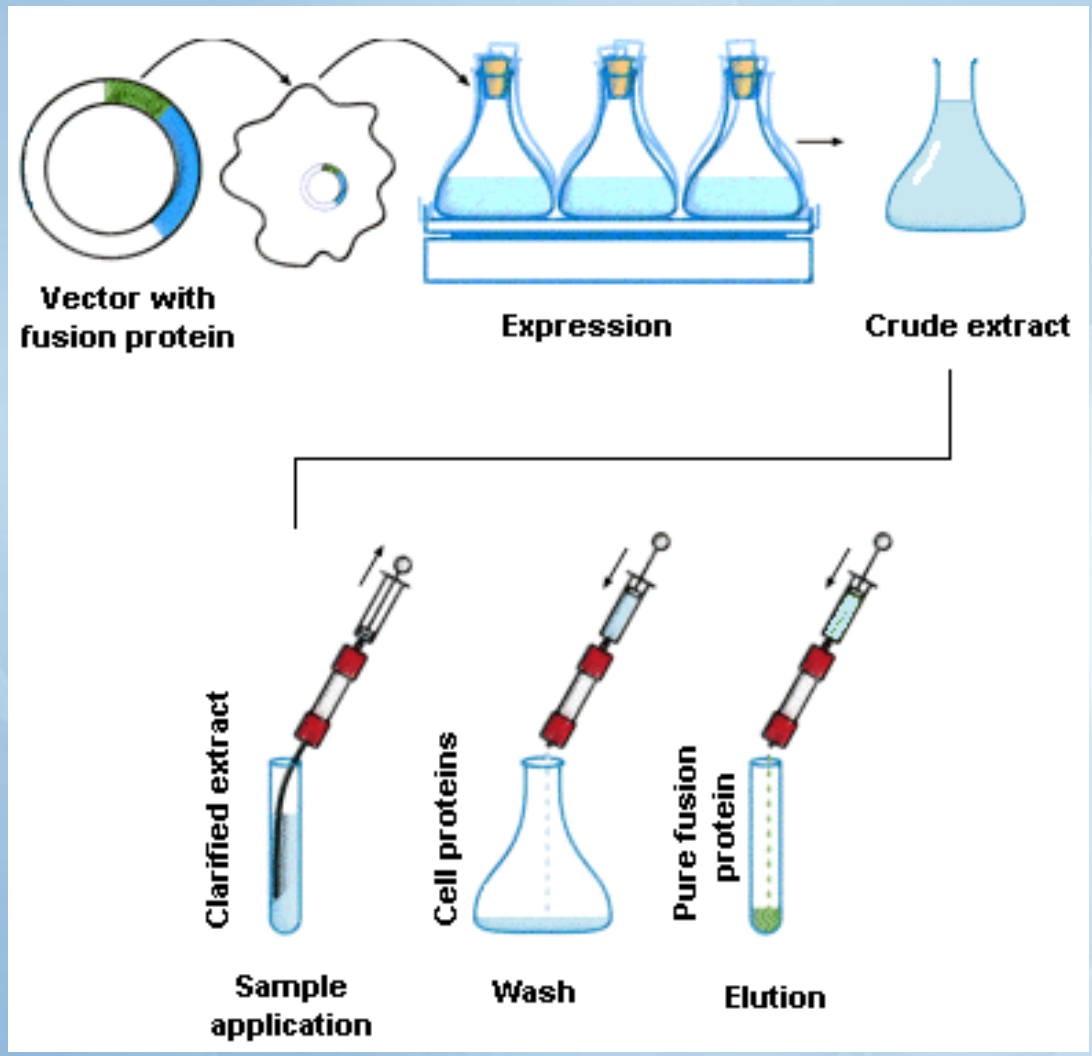
We want to work with pure proteins. How do we purify it from all the other *E. coli* proteins?

Affinity chromatography (AC)

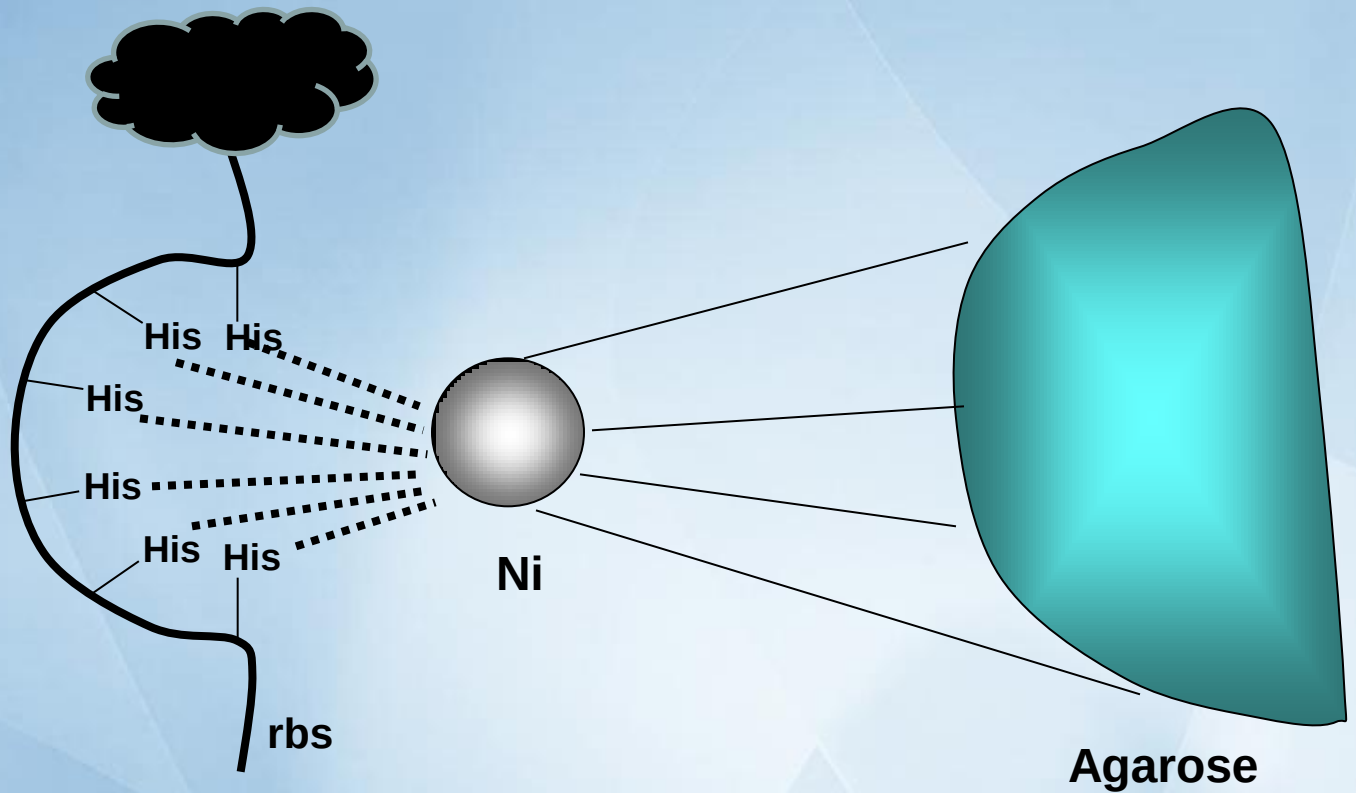
What is AC?

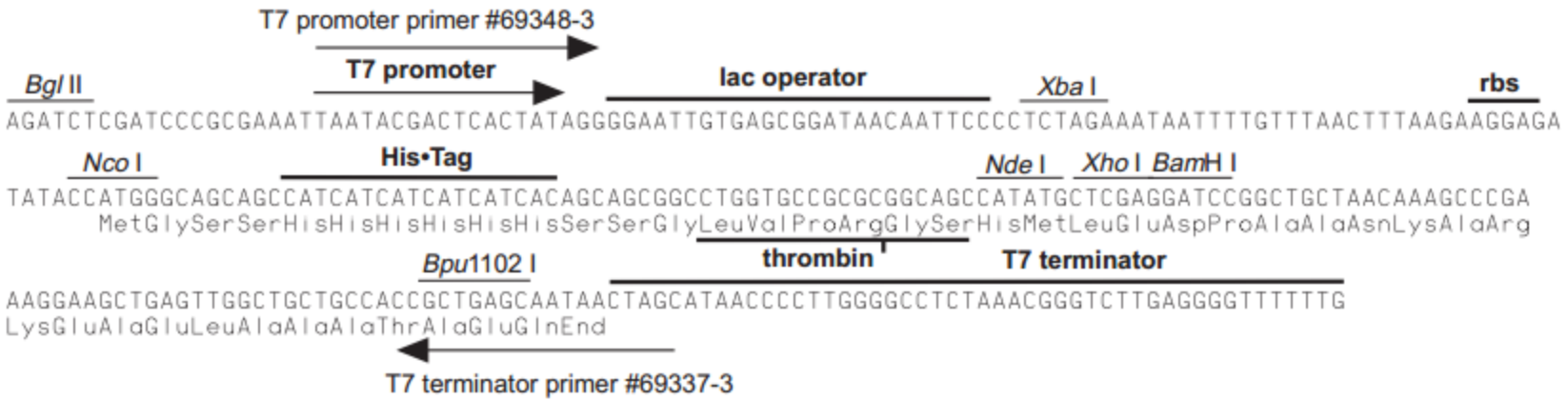
- **AC** is a technique enabling purification of a biomolecule with respect to biological function or individual chemical structure.
- AC is designed to purify a particular molecule from a mixed sample.

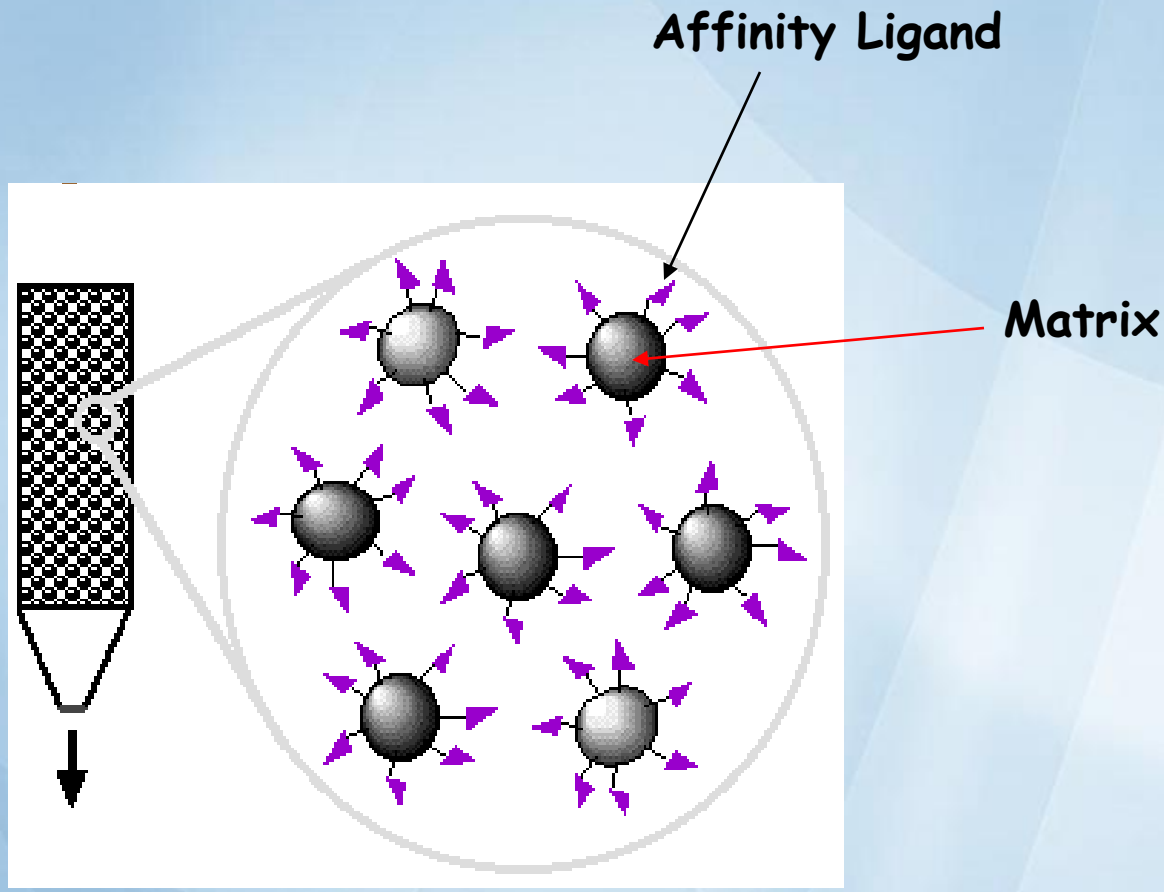
Affinity chromatography applied to recombinant proteins



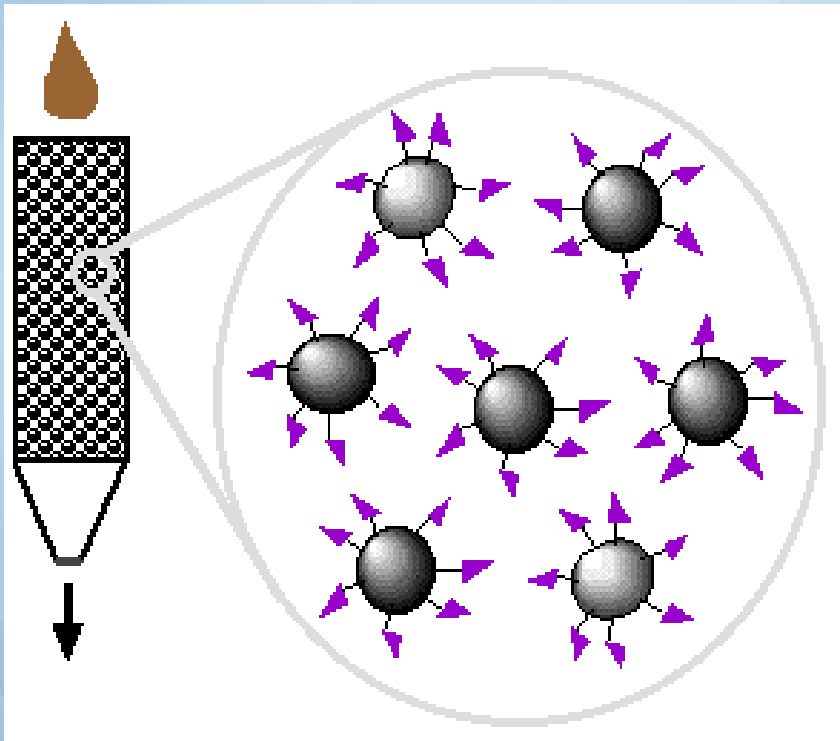
Affinity Chromatography



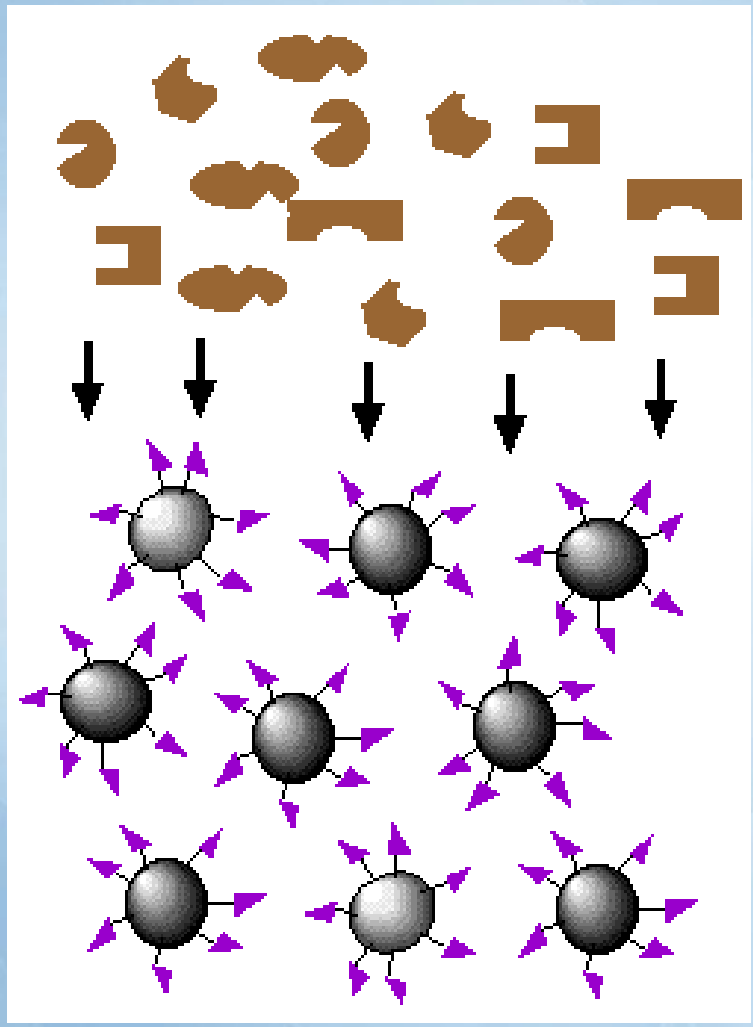



Affinity Ligand
Matrix

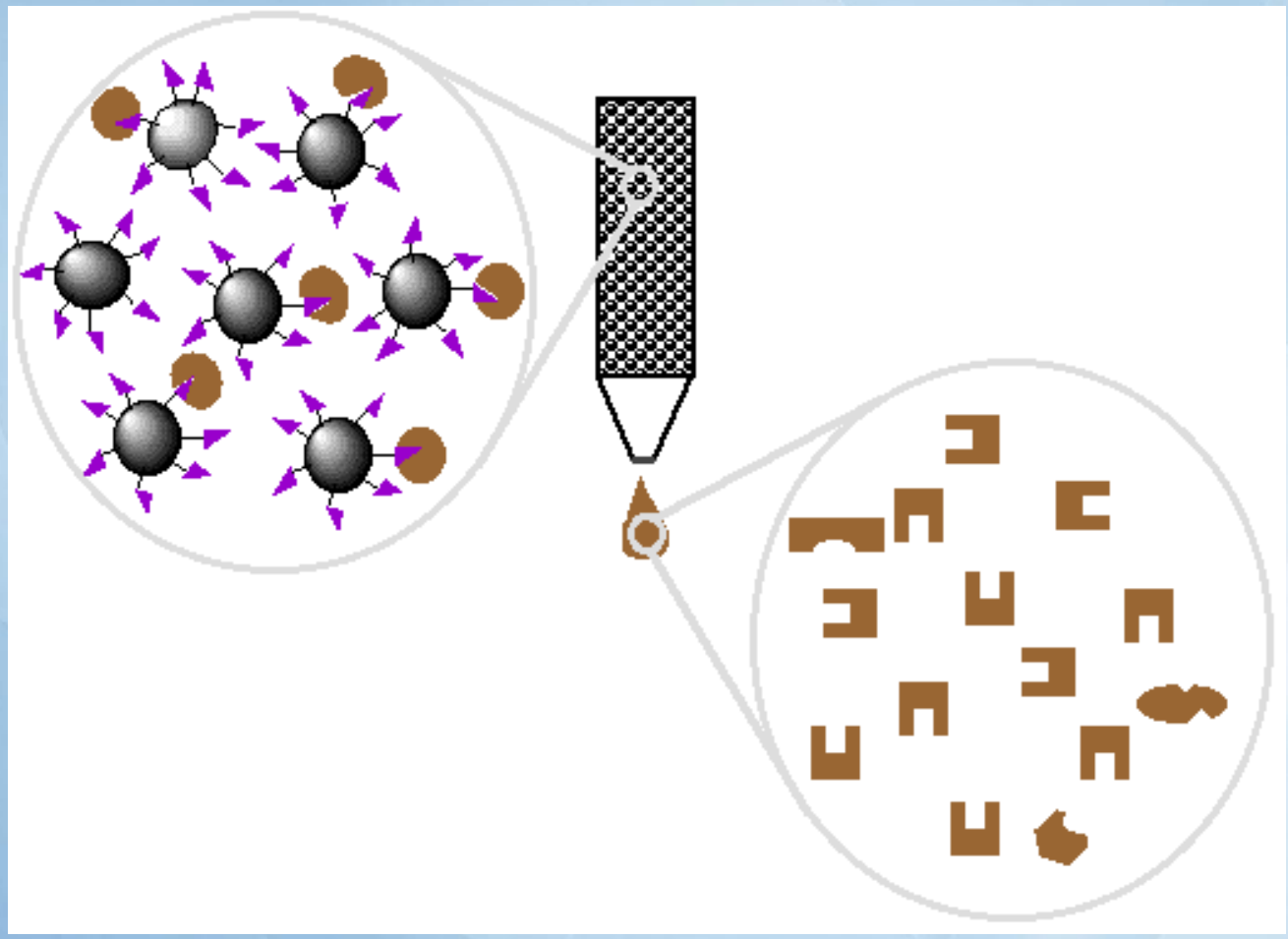
Step 1. Loading affinity column.



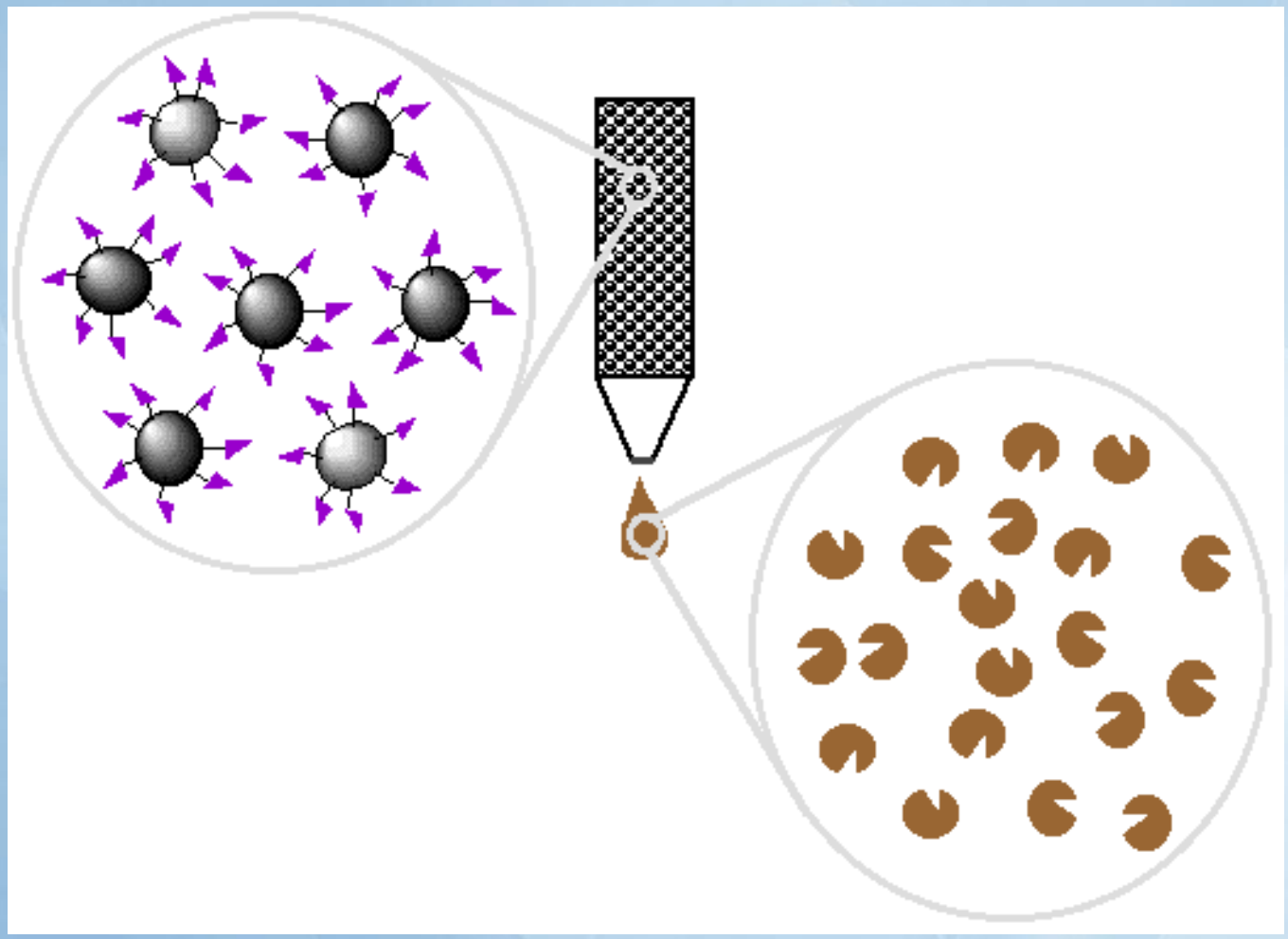
Step 2. Proteins sieve through matrix of affinity beads



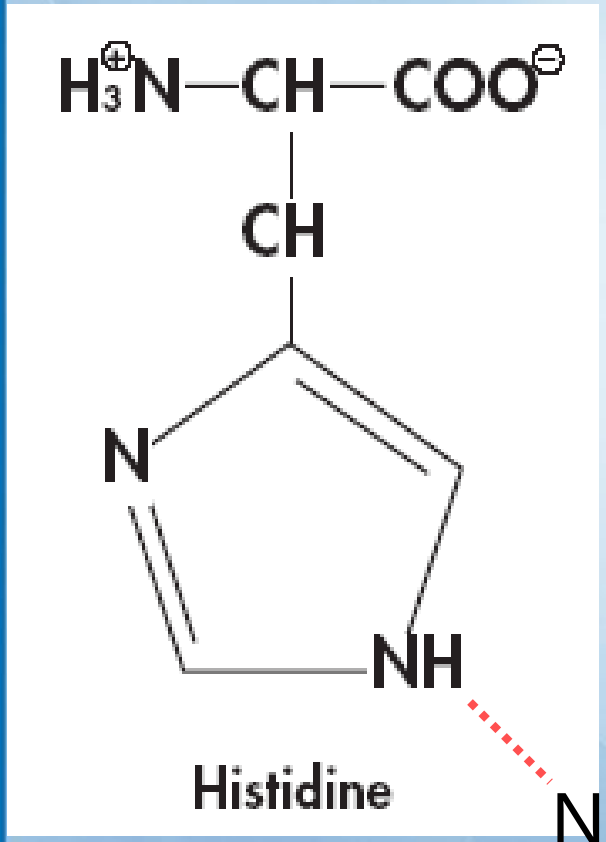
Step 5. Wash off proteins that bind loosely.



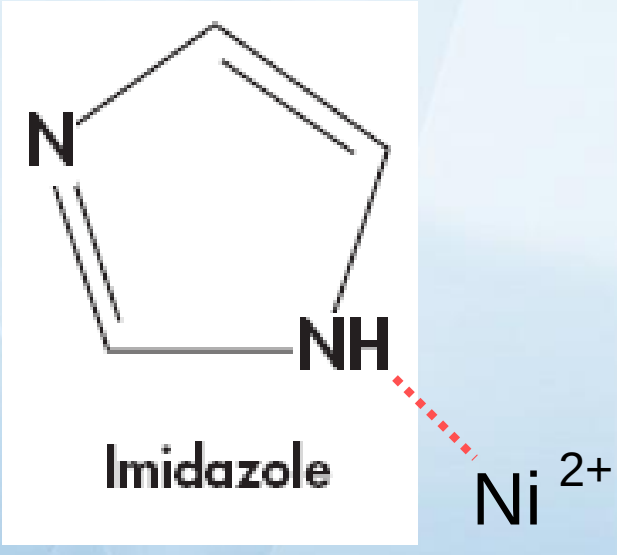
Step 6. Elute proteins that bind tightly to ligand and collect purified protein of interest.



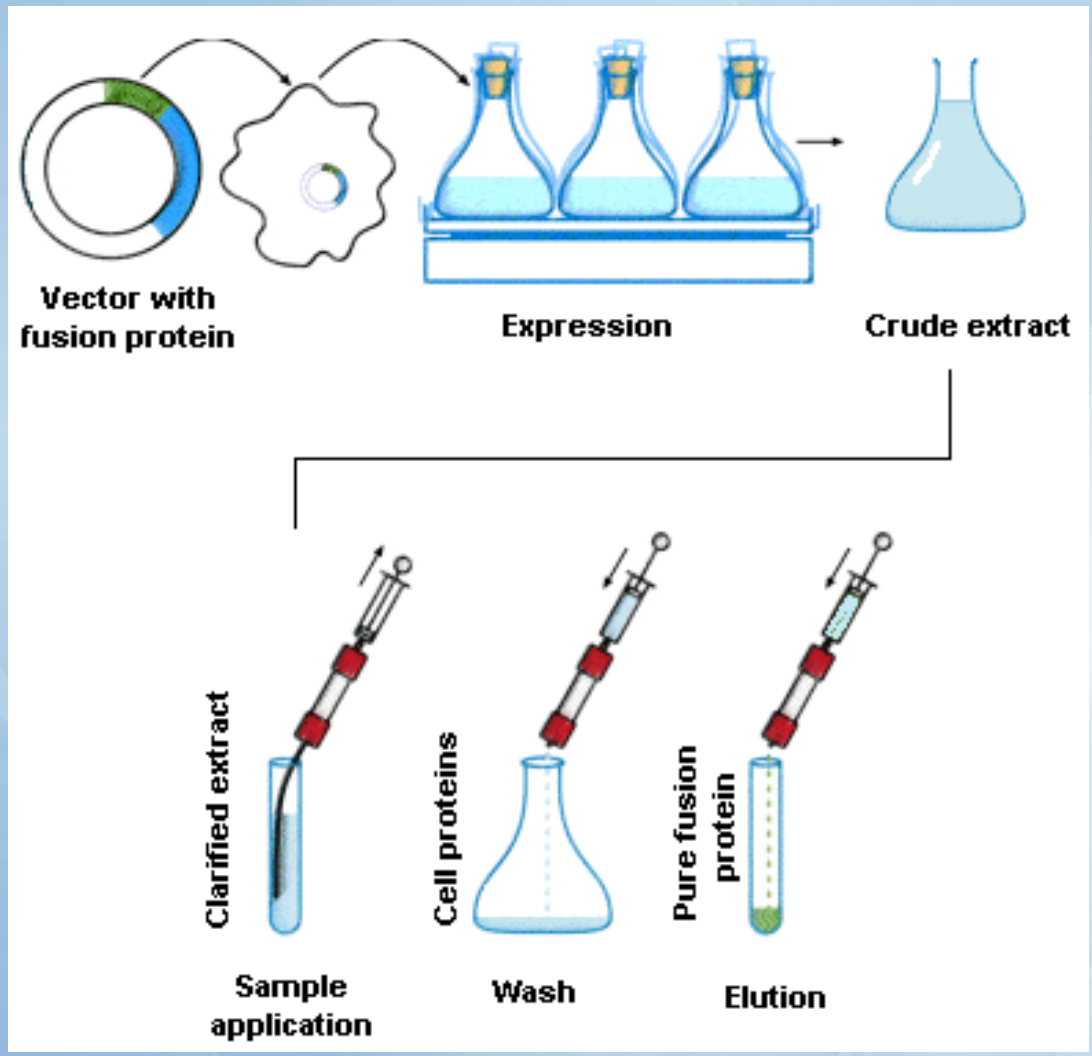
Elution with imidazole
Why imidazole?



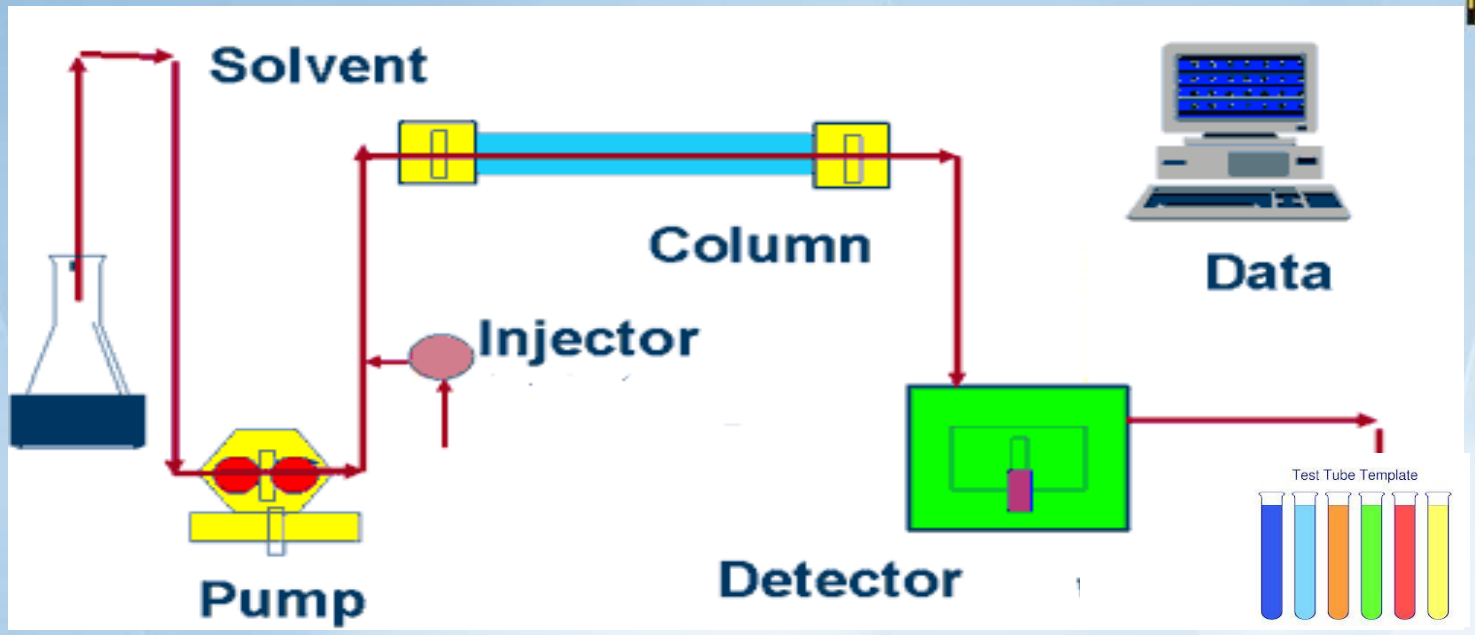
The imidazole ring is part of the structure of histidine

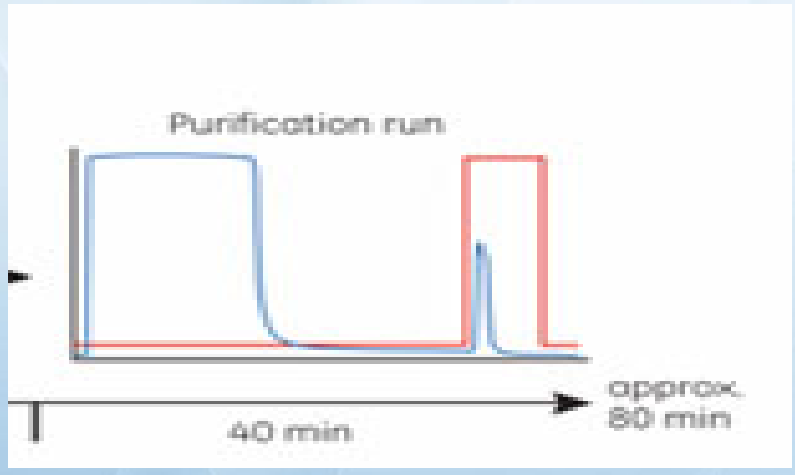


Affinity chromatography applied to recombinant proteins

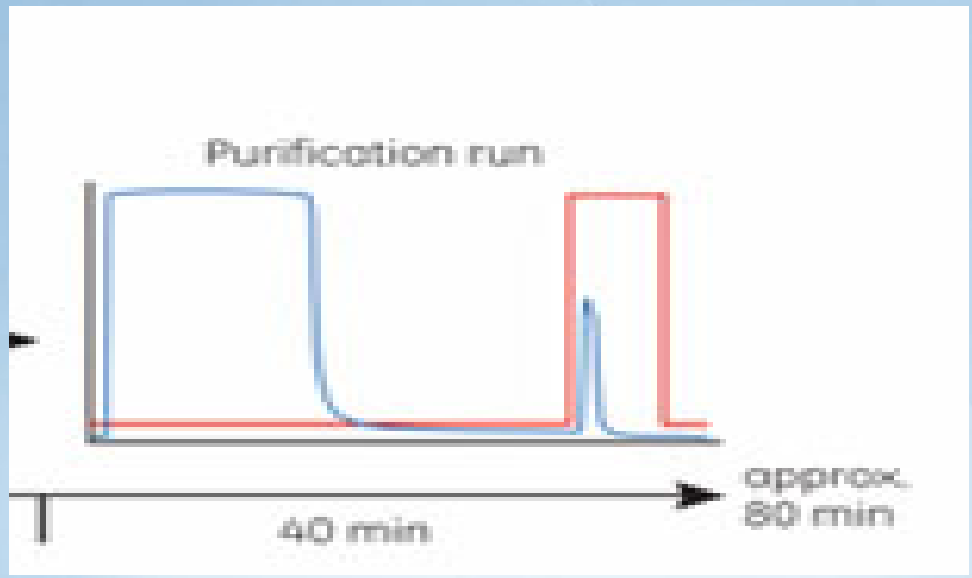


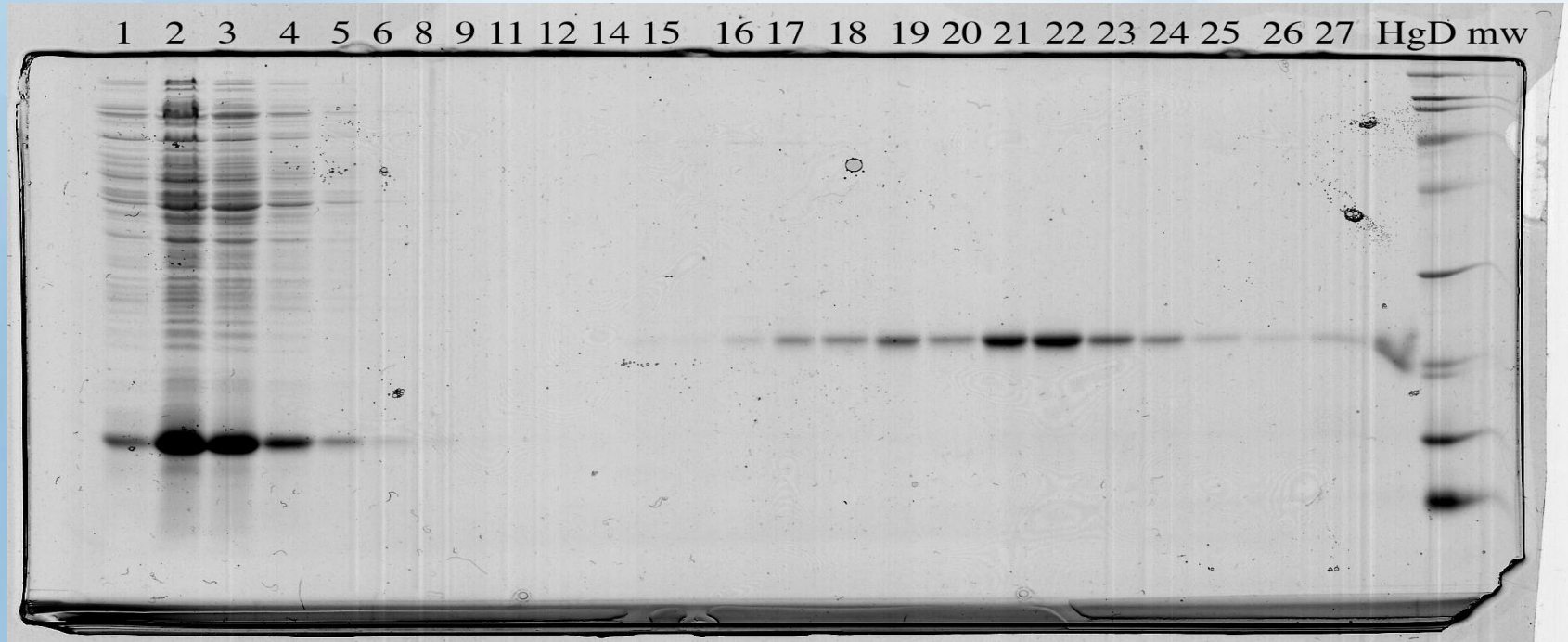
IMAC System


Solvent
Column
Data
Injector
Test Tube Template
Pump
Detector

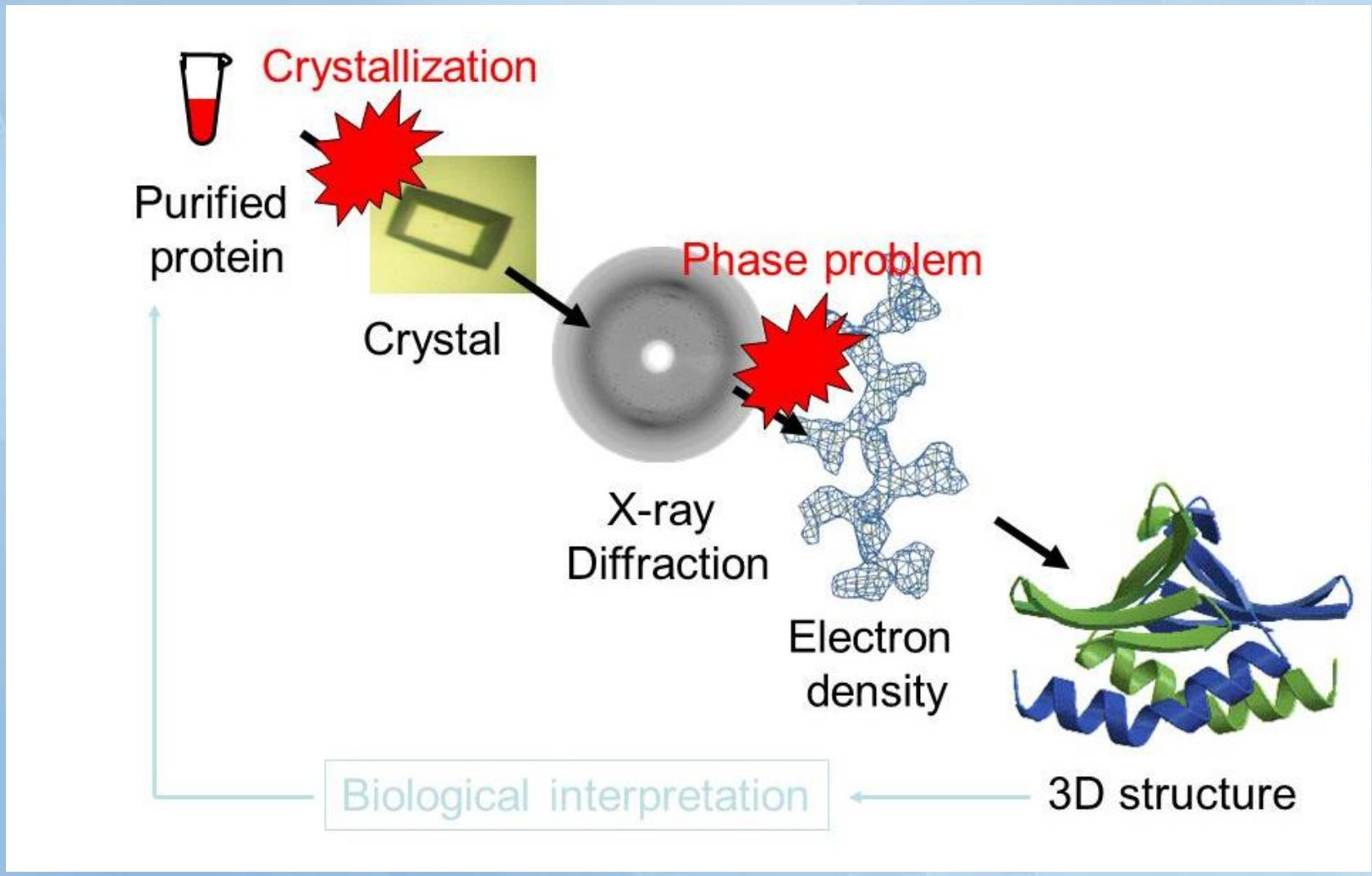

Purification run
approx.
80 min
40 min

Purity test

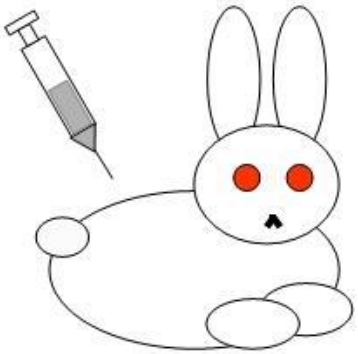

Purification run
approx.
80 min
40 min


1 2 3 4 5 6 8 9 11 12 14 15 16 17 18 19 20 21 22 23 24 25 26 27 HgD mw

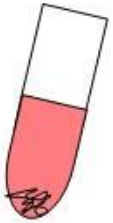
Protein structures



Produce Antibodies

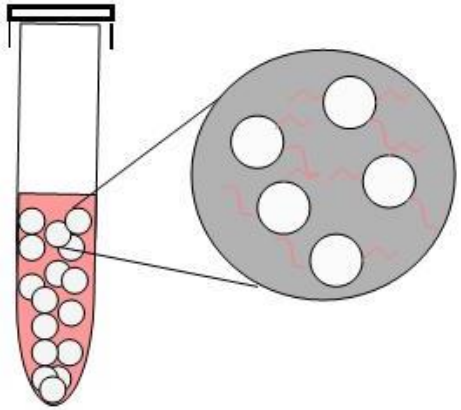


A solution containing a specific antigen is injected into a rabbit; the rabbit is immunized.



serum

Antiserum is taken from the rabbit; the supernatant contains the antibodies of interest.



Antibodies are incubated with sepharose beads that is conjugated to the original antigen.

Tools used for protein analysis

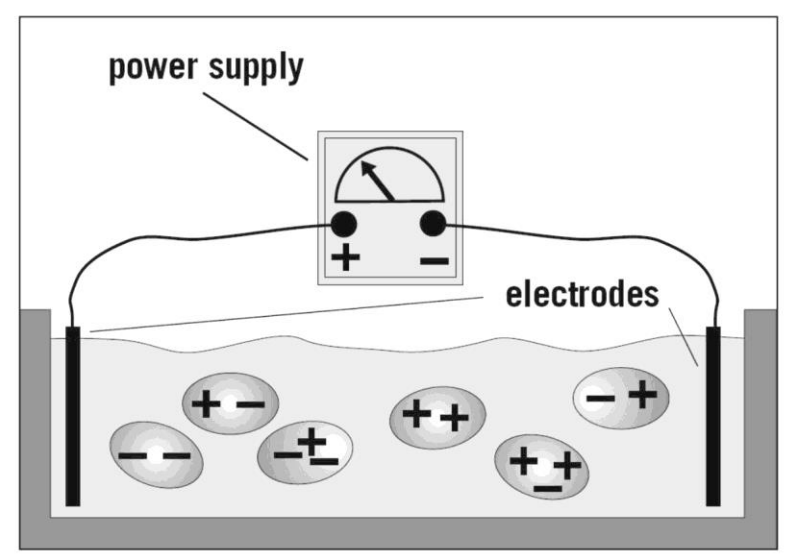
Electrophorasis

1. Native PAGE
2. Native Gradient PAGE
3. Urea PAGE
4. SDS PAGE
5. SDS Gradient PAGE
6. IEF
7. 2D PAGE
8. Western Blot

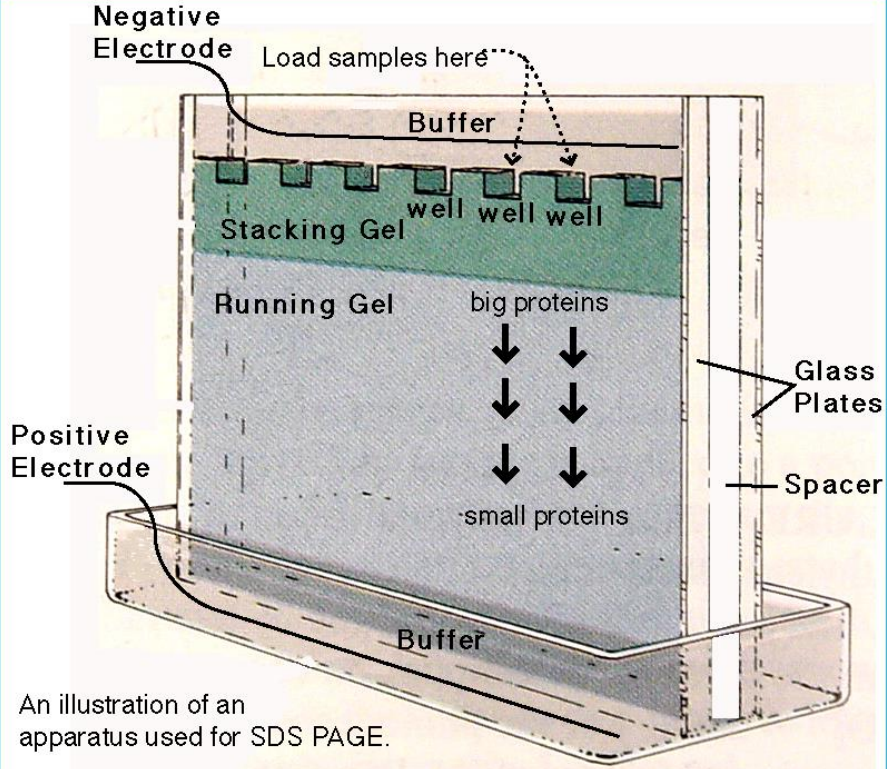
Without Electrophorasis

1. Cloning
2. Site-directed mutagenesis
3. protein tags
4. Protein structures
5. Proteomics
6. Protein Sequencing
7. Protein-protein interactions

SDS PAGE

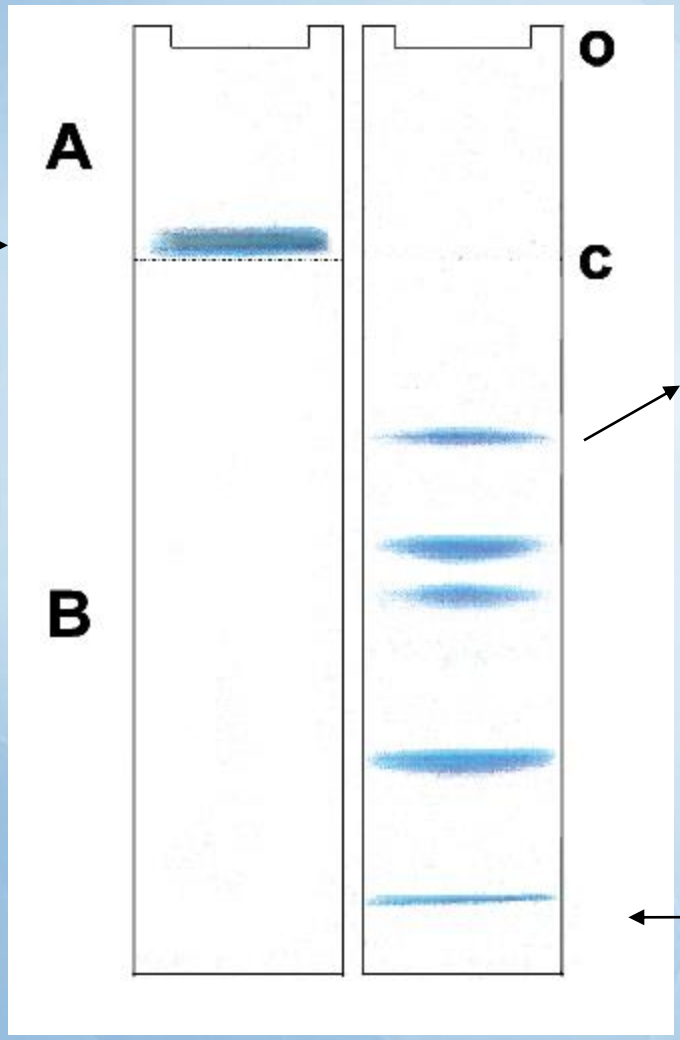


Proteins move in the electric field. Their relative speed depends on the charge, size, and shape of the protein



An illustration of an apparatus used for SDS PAGE.

Movement of Proteins on an SDS Gel



Protein Migration

-

Stacking of proteins at top of gel at start

Highest Molecular Wt. protein

Distribution of proteins in a charged field

+

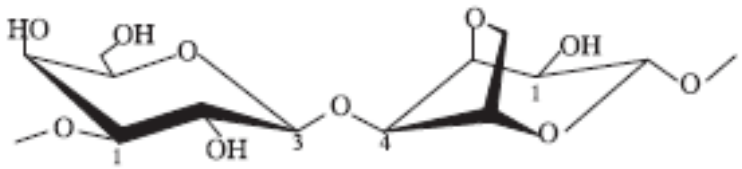
Low weight molecular dye

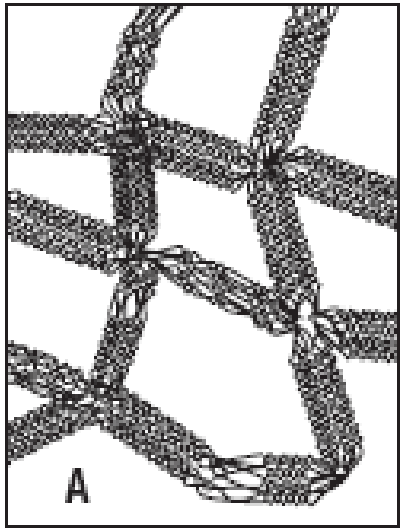
Gels

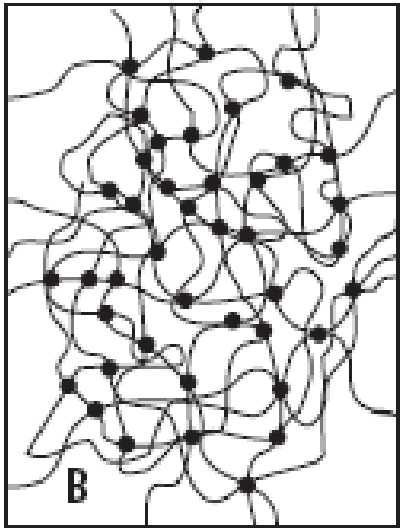
acrylamide

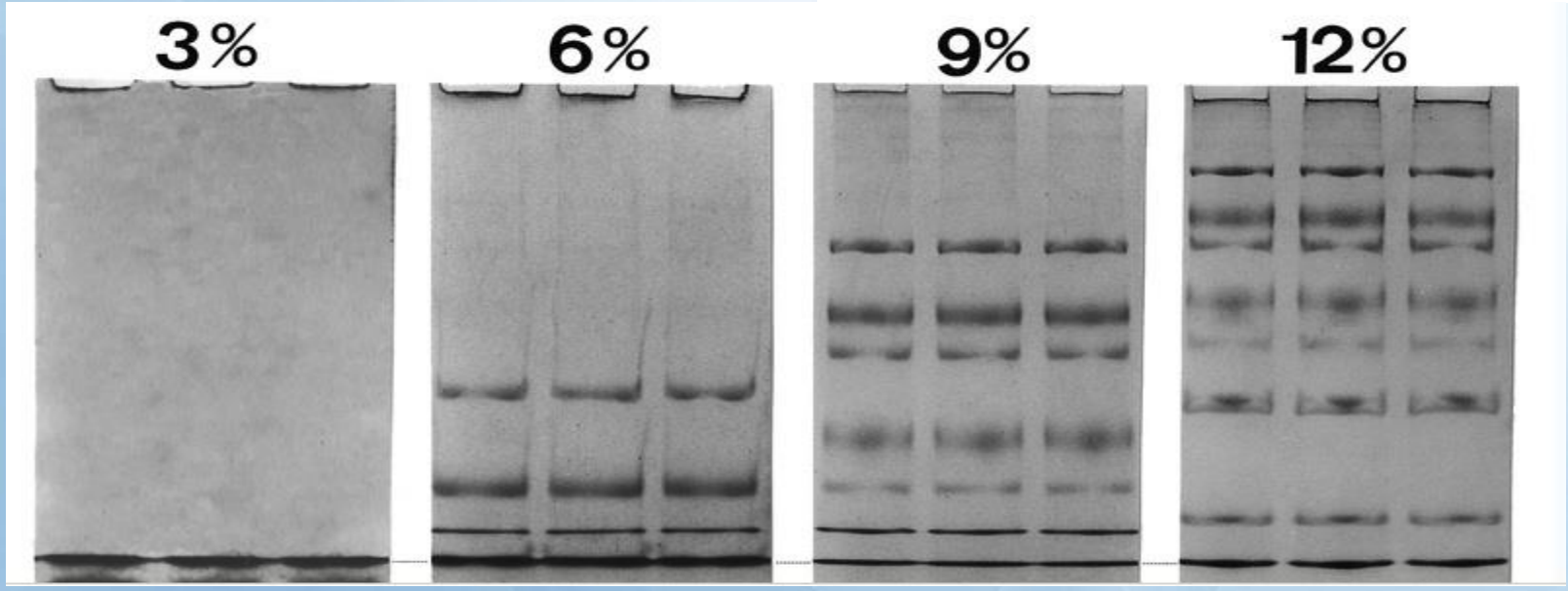
$$H_2C = CH\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH_2$$

agarose









Protein visualization on gels

Common stains:

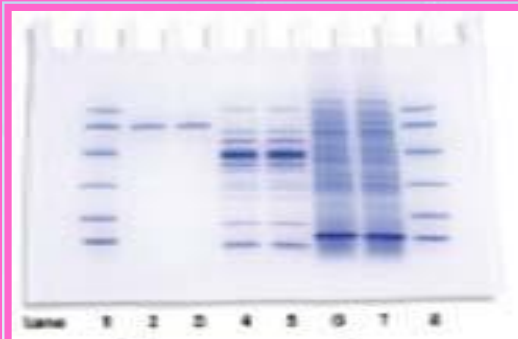
- **Coomassie Blue** in a fixative solution. Stain from a few hours to overnight. Destaining 4-12 hrs.

  * It provides a reasonably permanent record

- **Silver stain.** complex process, excellent, long-lasting record, sensitive.

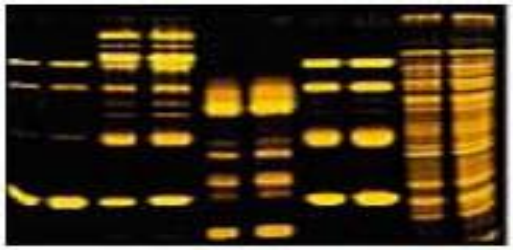
- **SYPRO (fluorescent)** staining is similar to Coomassie Blue in complexity, except the Destaining takes about 30 min.

  * It fades with time after a few hours



Coomassie

Silver

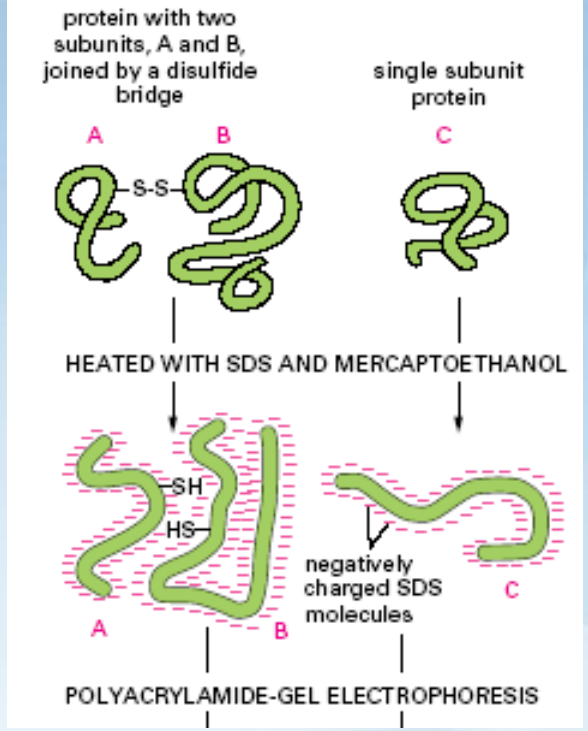


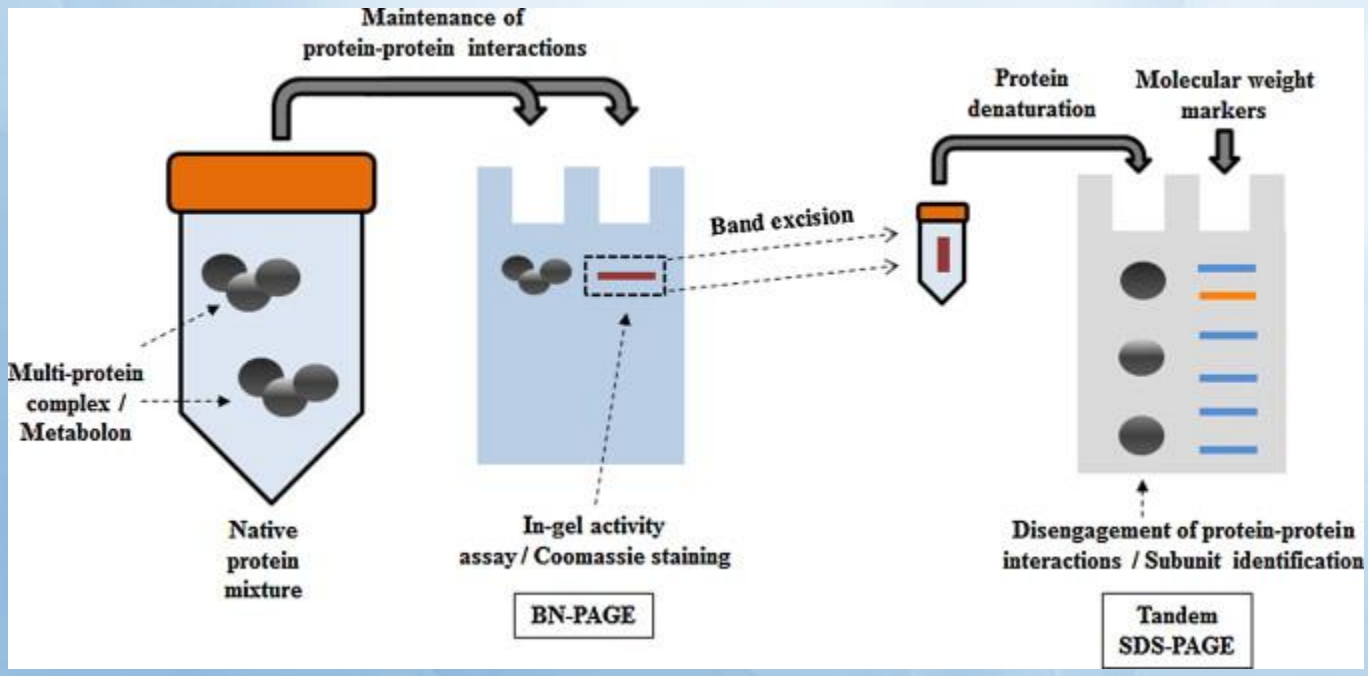
SYPRO orange

1. Native PAGE
2. Native Gradient PAGE
3. SDS PAGE
4. SDS Gradient PAGE
5. IEF
6. 2D PAGE
7. Western Blot

Native PAGE

Non-denatured protein

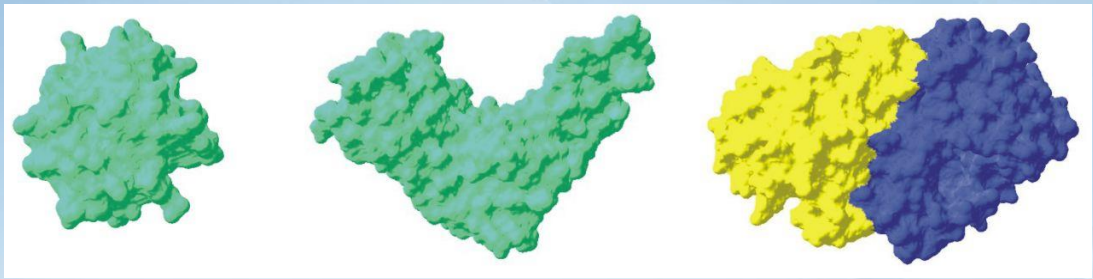


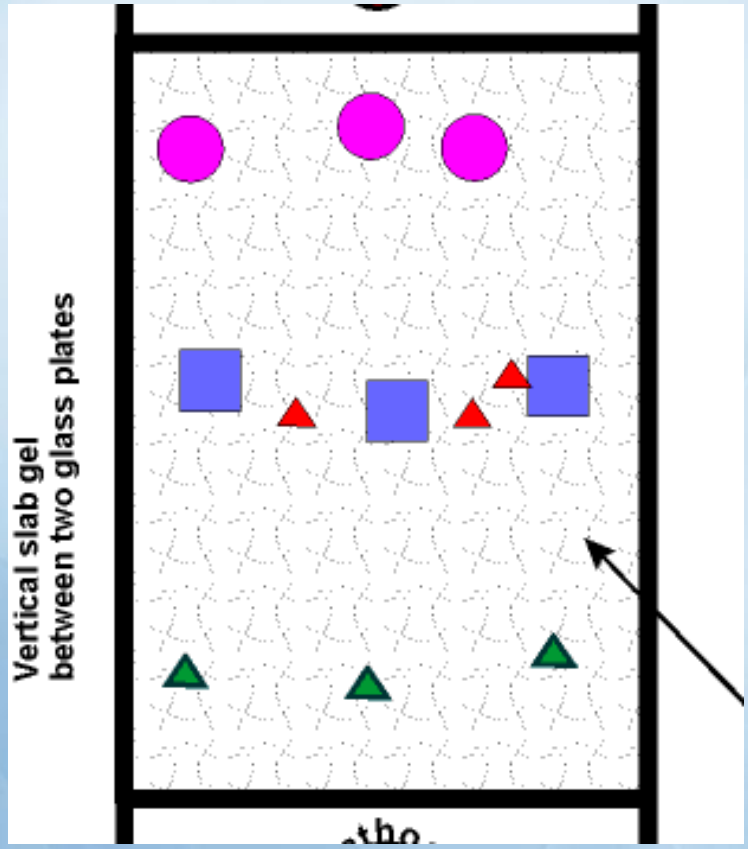


Native PAGE

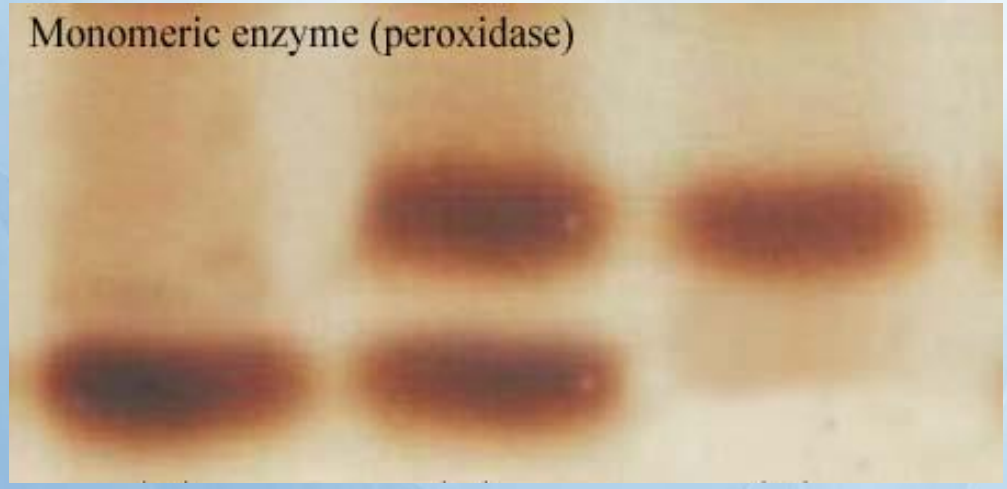
Separates by

- charge
- size
- shape

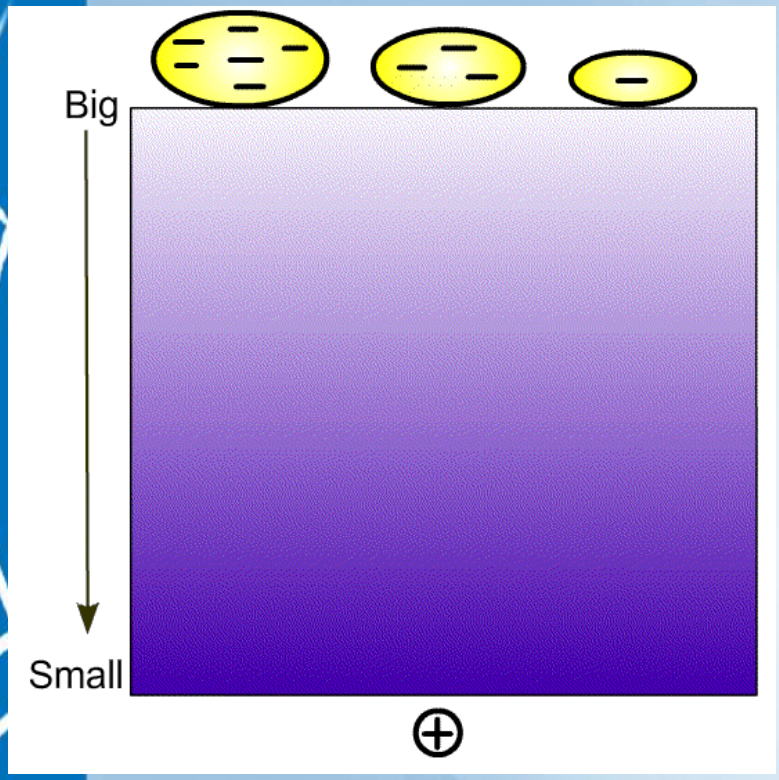


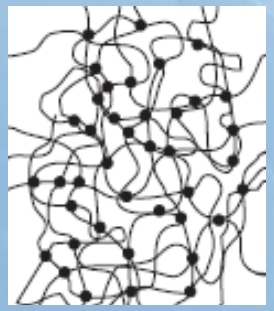


- reaction with specific activity stains (depending on enzyme).
  - substrates + cofactors + stain + buffer
- colored bands such as Est, Prx, Mdh …
- Colorless bands (white bands on a dark background, negatively stained) such as SOD.



Native gradient PAGE

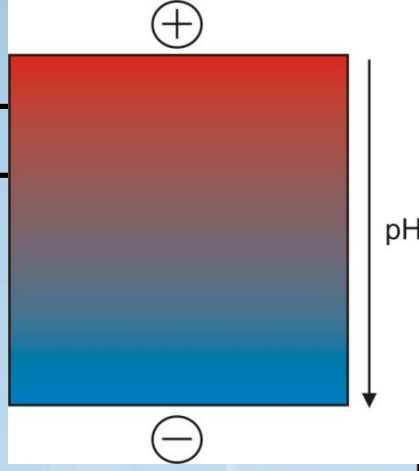




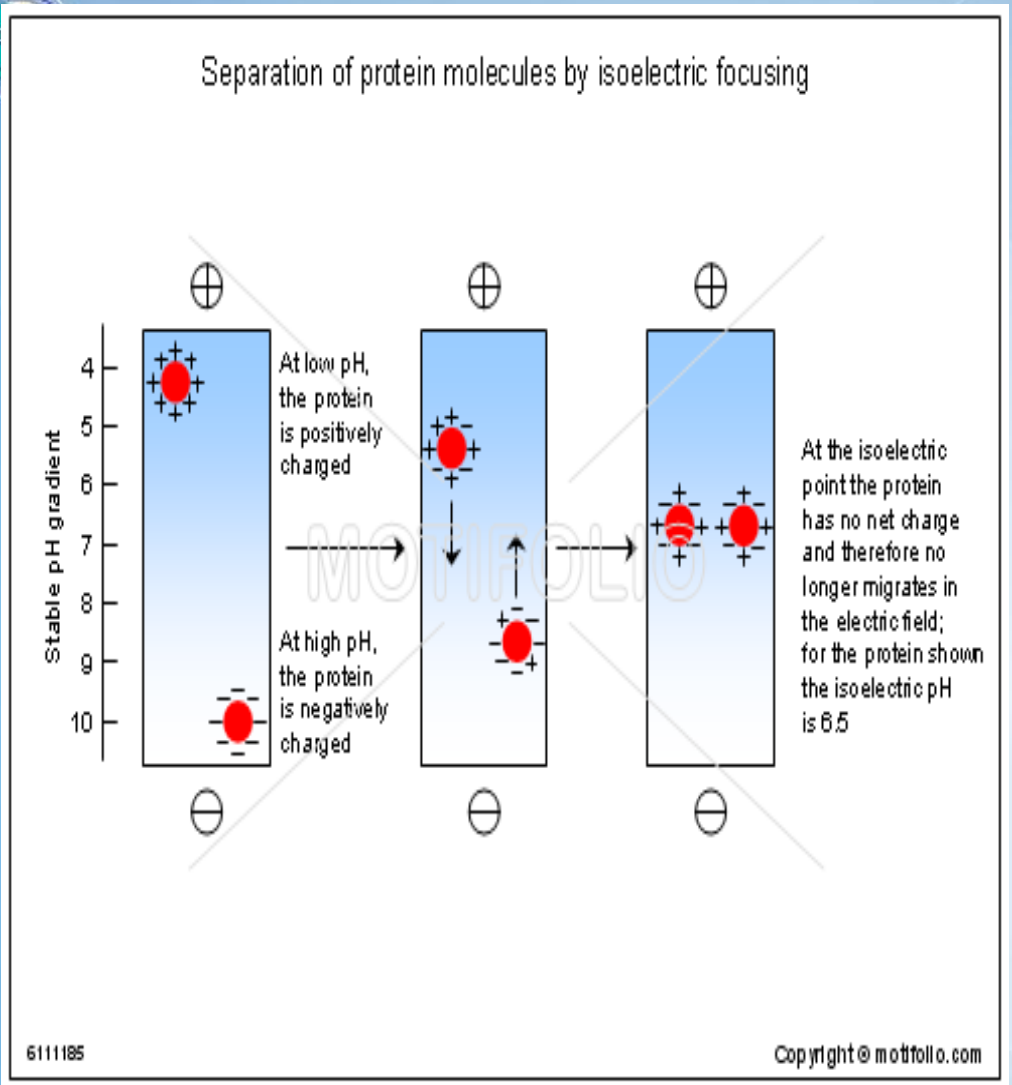
Separate native proteins by size – proteins stop moving when they reach a sertain gel density (but this may take a very long time ...)

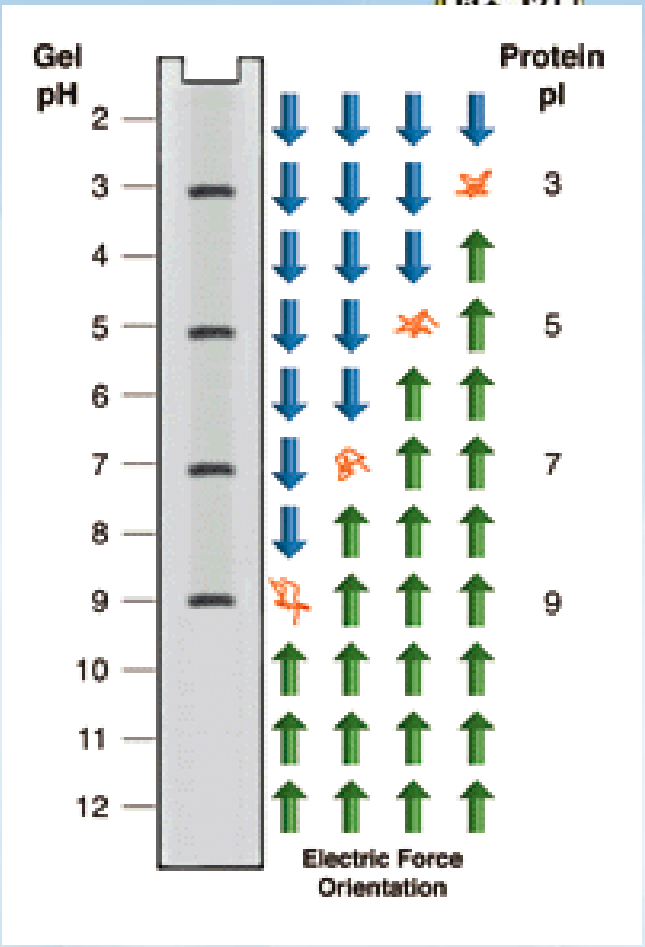
A great technique to study protien oligomerization!

What is Isolectric focusing?



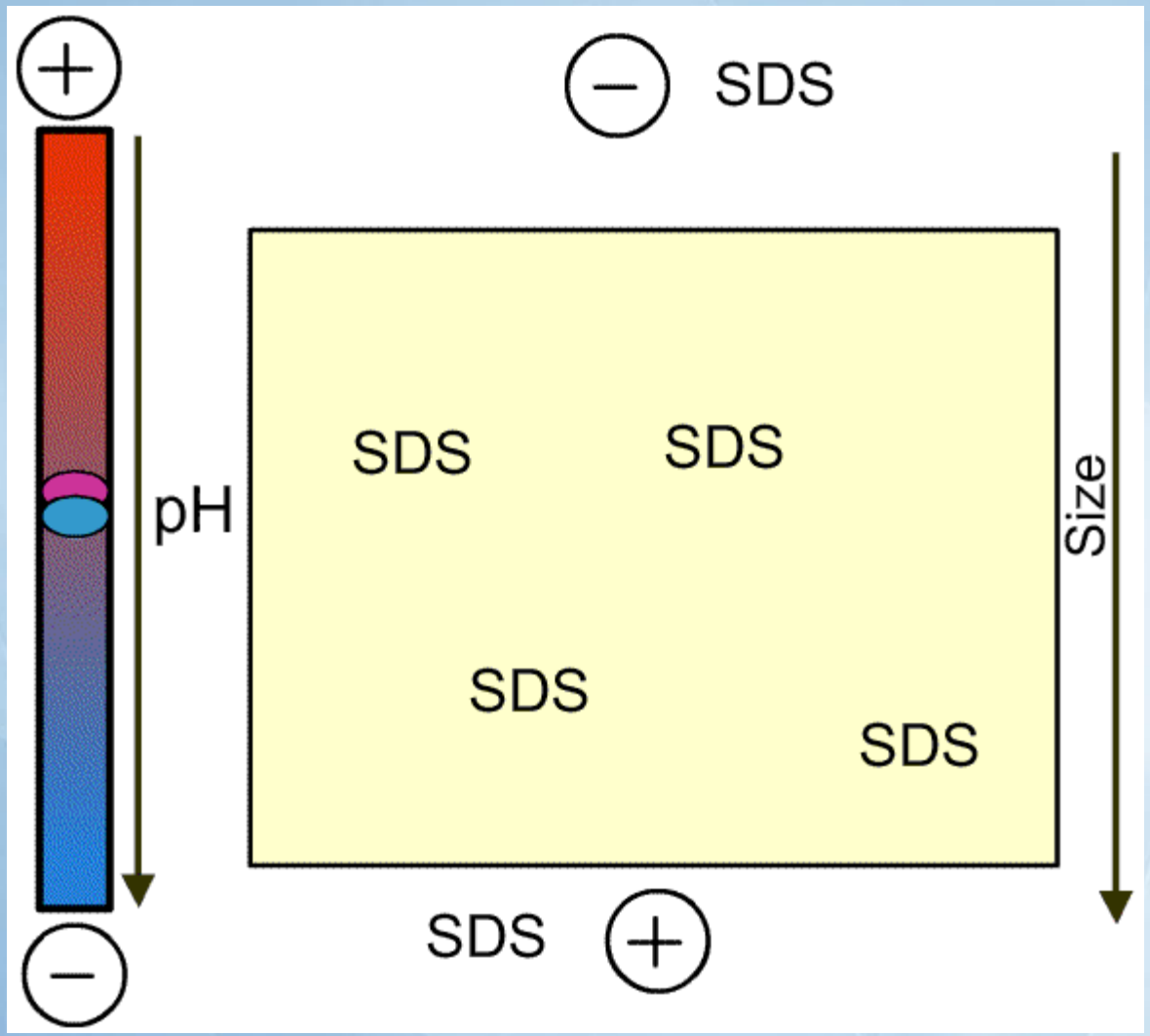
- Gel is prepared with pH gradient
- Separates proteins by their isoelectric points (pI)
- Each protein has own pI = pH at which the protein has equal amount of positive and negative charges (the net charge is zero)
- Charge on the protein changes as it migrates across pH
- When it gets to pI, has no charge and stops



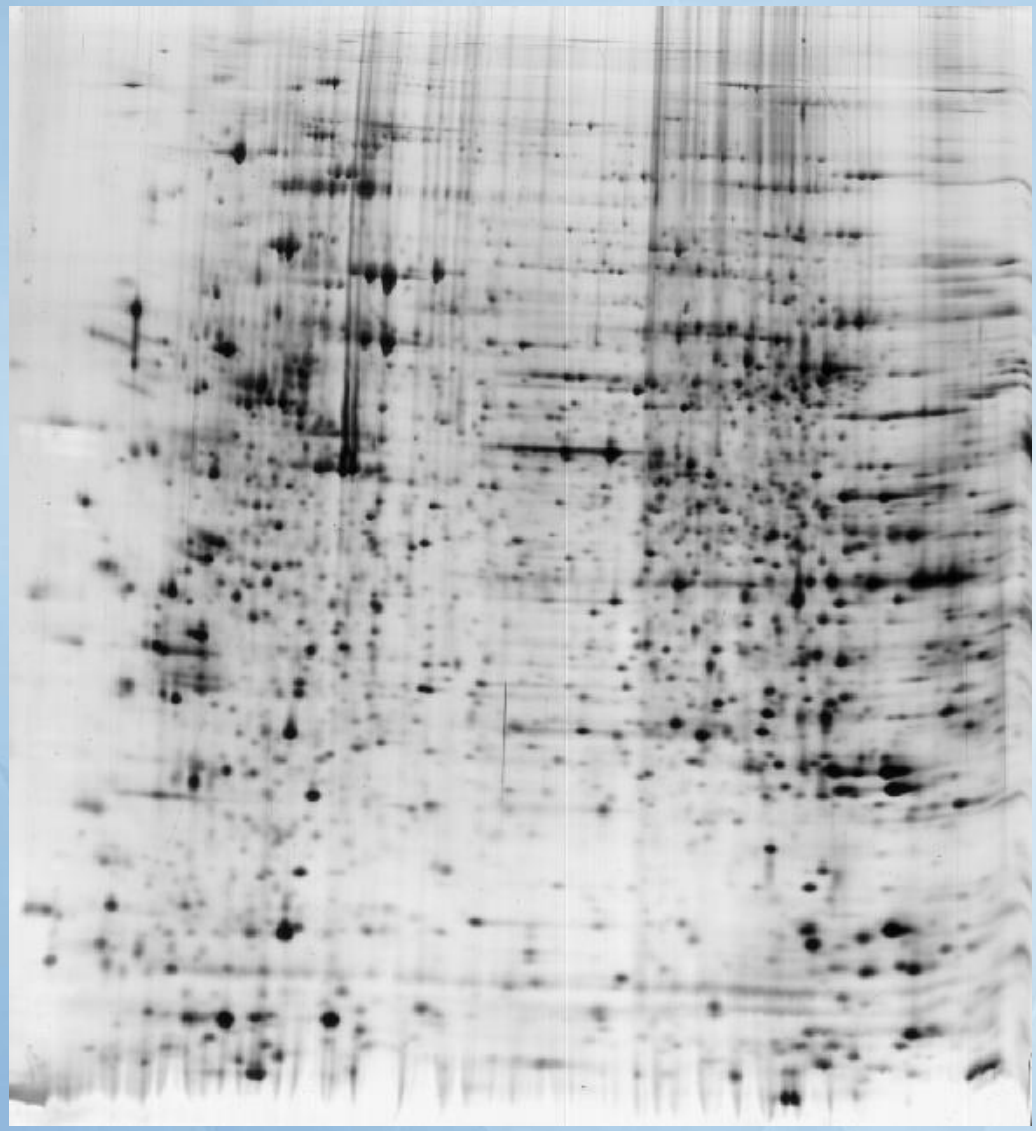


- PI of proteins can be theoretically predicted. Therefore, IEF can also be used for protein identification.

2D PAGE



2D PAGE



Western Blot Analysis

- Identifies protein through antibody interaction
- Run proteins on denatured gel (SDS-PAGE)
- Transfer (blot) proteins onto membrane
- Probe the membrane with primary antibody
- Add secondary antibody (this antibody is linked to an enzyme)
- Substrate is added and color appears