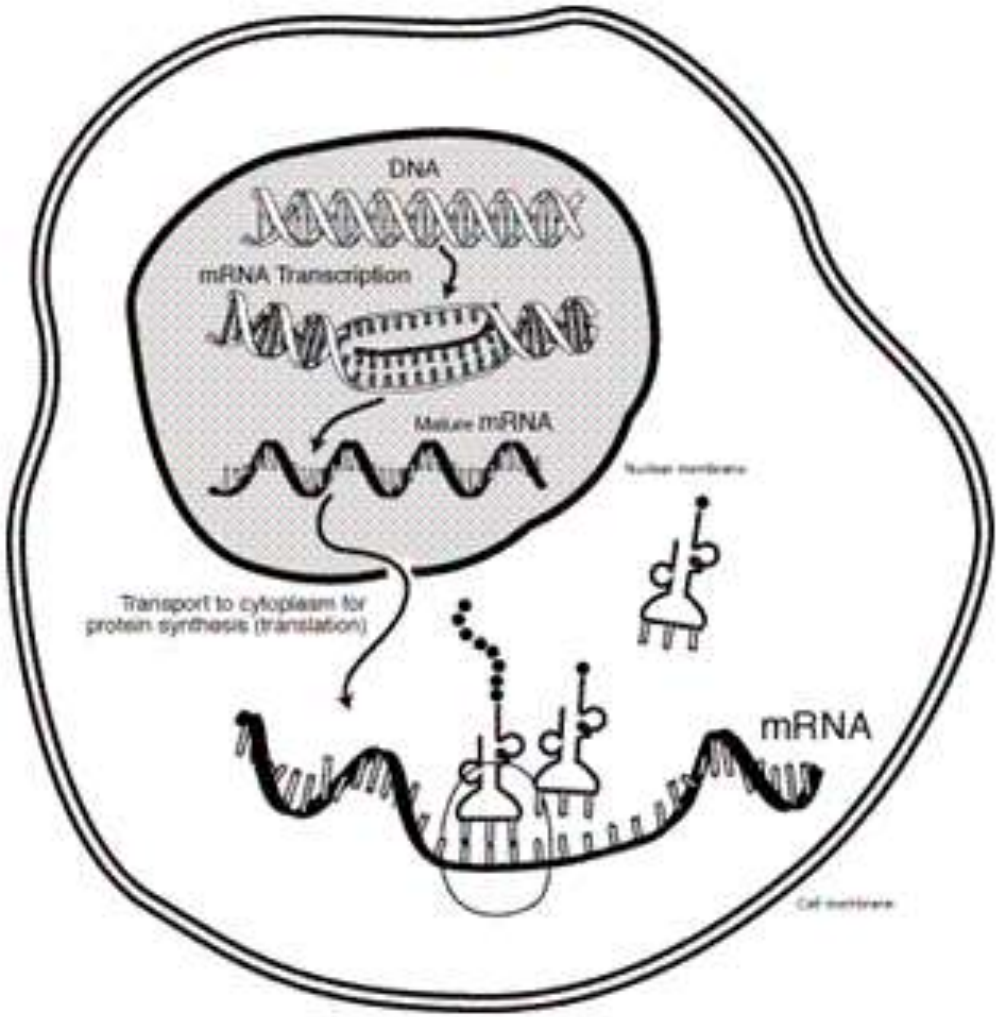
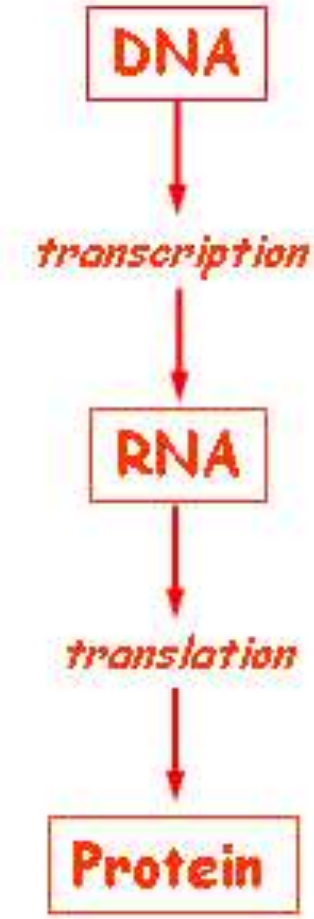
Protein Expression and Purification

Central Dogma of Molecular Biology





Proposed by Francis Crick, 1958

Expression vector

- The expression vector, otherwise known as an expression construct, is usually a plasmid or virus designed for protein expression in cells
- The expression vector is a plasmid engineered to introduce a particular gene into the target cell

Protein expression in *E. coli*

pGEX plasmid:

- Gene encoding affinity tag-glutathione S tranferase (GST)
- Spacer between genes - encodes protease cleavage site (thrombin)
- P_{tac} promoter-induce with IPTG
- Ribosome binding site

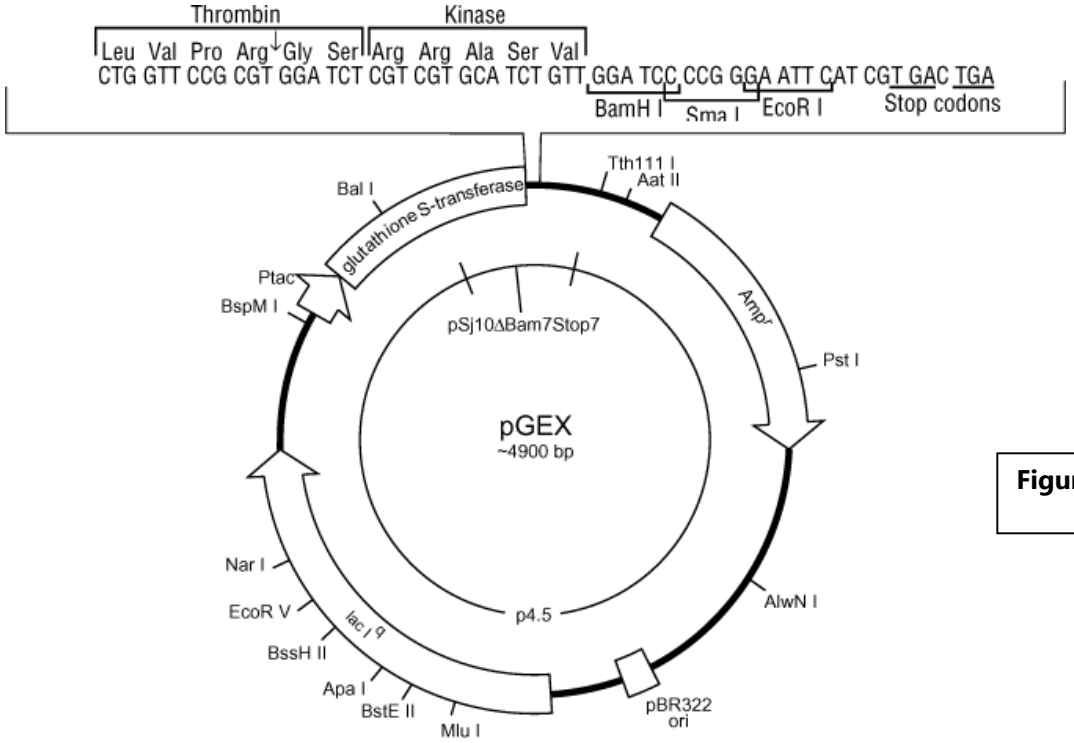
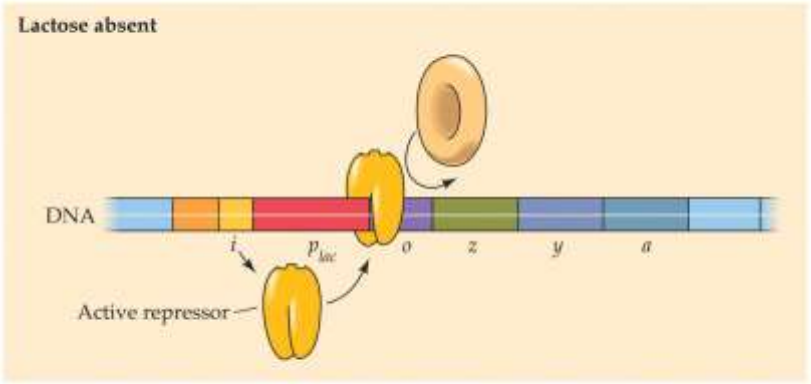
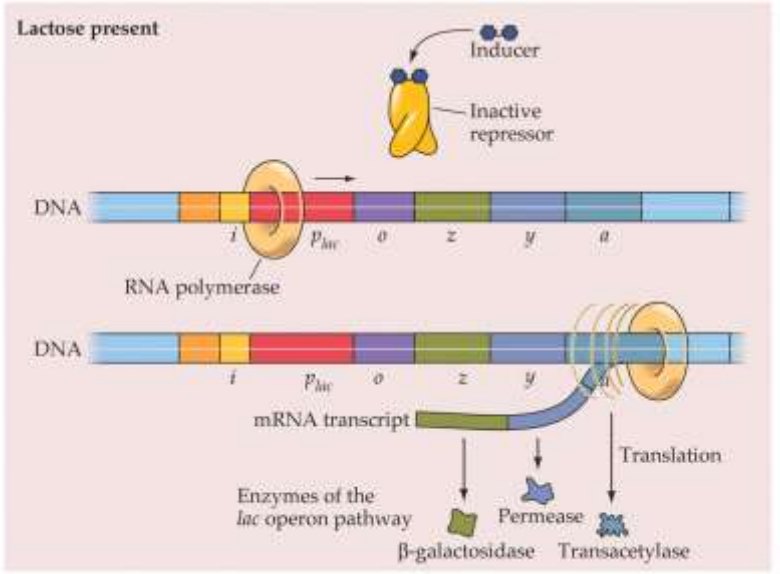


Figure 1: Diagram of the pGEX expression vector.

IPTG-inducible protein expression

Isopropyl β-D-1-thiogalactopyranoside





Ligation inserts gene in-frame with GST

In frame in pGEX-2T

```
                    BamHI
CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG
 L   V   P   R   G   S   P   G   I   H   R   D   *
```

Insert into BamHI site

```
                    BamHI              insert          BamHI
CTG GTT CCG CGT GGA TCC CTG GGT GAG CGT GAA GCG GGA TCC CCG GGA ATT CAT CGT GAC TGA
 L   V   P   R   G   S   L   G   E   R   E   A   G   S   P   G   I   H   R   D   *
```

Out of frame in pGEX-3X

```
                      BamHI
ATC GAA GGT CGT GGG ATC CCC GGG AAT TCA TCG TGA CTG ACT GAC
 I   E   G   R   G   I   P   G   N   S   S   *
```

Insert into BamHI site

```
                    BamHI              insert          BamHI
ATC GAA GGT CGT GGG ATC CCT GGG TGA GCG TGA AGC GGG ATC CCC GGG AAT TCA TCG TGA
 I   E   G   R   G   I   P   G   *   A   *   S   G   I   P   G   N   S   S   *
```

* indicates stop codon

Why purify a protein?

- To study its function
- To analyze its physical properties
- To determine its sequence
- For industrial or therapeutic applications

Steps in Recombinant Protein Purification

1. Design expression plasmid, transform, select
2. Grow culture of positive clone, induce expression
3. Lyse cells
4. Centrifuge to isolate protein-containing fraction
5. Column Chromatography—collect fractions
6. Assess purity on SDS-PAGE

Why bacteria for protein expression

Bacterial Genetics

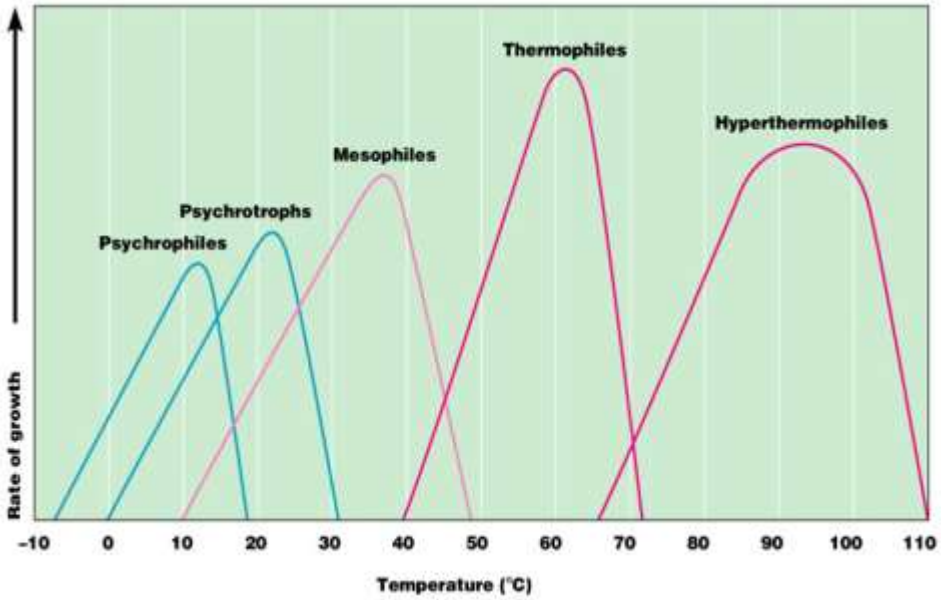
- Bacteria grow and divide rapidly
  - Divide every 20 minutes or so
  - Millions of cells can be grown on small dishes of agar or in liquid culture media
- Easy-to-make mutant strains to be used for molecular and genetic studies

Microbial growth = increase in number of cells, not cell size

The Requirements for Growth:

1. Physical Requirements

- Temperature range where organism can grow:
  - Minimum growth temperature
  - Optimum growth temperature (best range)
  - Maximum growth temperature



Physical Requirements:

pH

- Most bacteria grow between pH 6.5 and 7.5.
- Molds and yeasts grow between pH 5 and 6.
- Acidophiles grow in acidic environments (coal mines).

Osmotic Pressure

- Hypertonic environments, increase salt or sugar, cause plasmolysis. It inhibits cell growth
  - This phenomenon is used to stop food spoilage.
- Extreme or obligate halophiles require high osmotic pressure since they live in high salt concentrations.
- Facultative halophiles can tolerate high osmotic pressure, up to 15% salt.
- Most microorganisms grow in a medium that is nearly all water.

- **The Requirements for Growth:**

2.Chemical Requirements

- Carbon: backbone of living matter
  - Structural organic molecules, energy source
  - Chemoheterotrophs use organic carbon sources energy from protein, carbohydrates and lipids
  - Autotrophs get it from CO_2
- Nitrogen: also needed for synthesis of cellular material
  - In amino acids, proteins
  - Most bacteria decompose proteins
  - Some bacteria derive N from NH_4^+ or NO_3^-
  - A few bacteria use N_2 in nitrogen fixation directly from atmosphere

Chemical Requirements

- Sulfur
  - used to synthesize sulfur-containing amino acids and vitamins (thiamine, biotin)
- Phosphorus
  - used to synthesize nucleic acids and phospholipids
  - In DNA, RNA, ATP, and membranes
  - Phosphate ion, PO_4^{3-} is a source of phosphorus
- Trace Elements
  - Iron, copper, molybdenum and zinc; (Inorganic elements required in small amounts)
  - Usually work as enzyme cofactors
- Organic Growth Factors
  - Organic compounds that are only obtained from the environment
  - Vitamins, amino acids, purines, pyrimidines

Culture Media

- Culture Medium: Nutrients prepared for microbial growth
- Sterility: No living microbes
- Inoculum: Introduction of microbes into medium
- Culture: Microbes growing in/on culture medium

Agar

- Complex polysaccharide
- Used as solidifying agent for culture media in Petri plates, slants, and deeps
- Generally not metabolized by microbes
- Liquefies at 100°C
- Solidifies ~40°C

Culture Media

- **Chemically Defined Media:** Exact chemical composition is known. The medium must contain organic growth factors that serve as a source of carbon and energy
  - Known for growth of "fastidious" and autotrophic organism
  - Used in tests to determine concentration of a vitamin in a substance

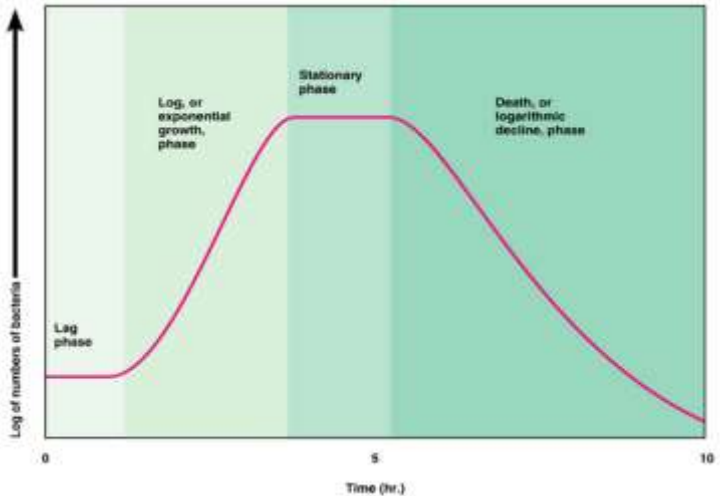
- **Complex Media:** Extracts and digests of yeasts, meat, or plants
- Nutrient broth: liquid
- Nutrient agar: Solid

- **Preserving Bacteria Cultures**
- **Deep-freezing:** -50°to -95°C (30%Glycerol is added to the culture the cells)

- **Lyophilization (freeze-drying):** Frozen (-54° to -72°C) and dehydrated in a vacuum (sublimation)

Phases of Growth

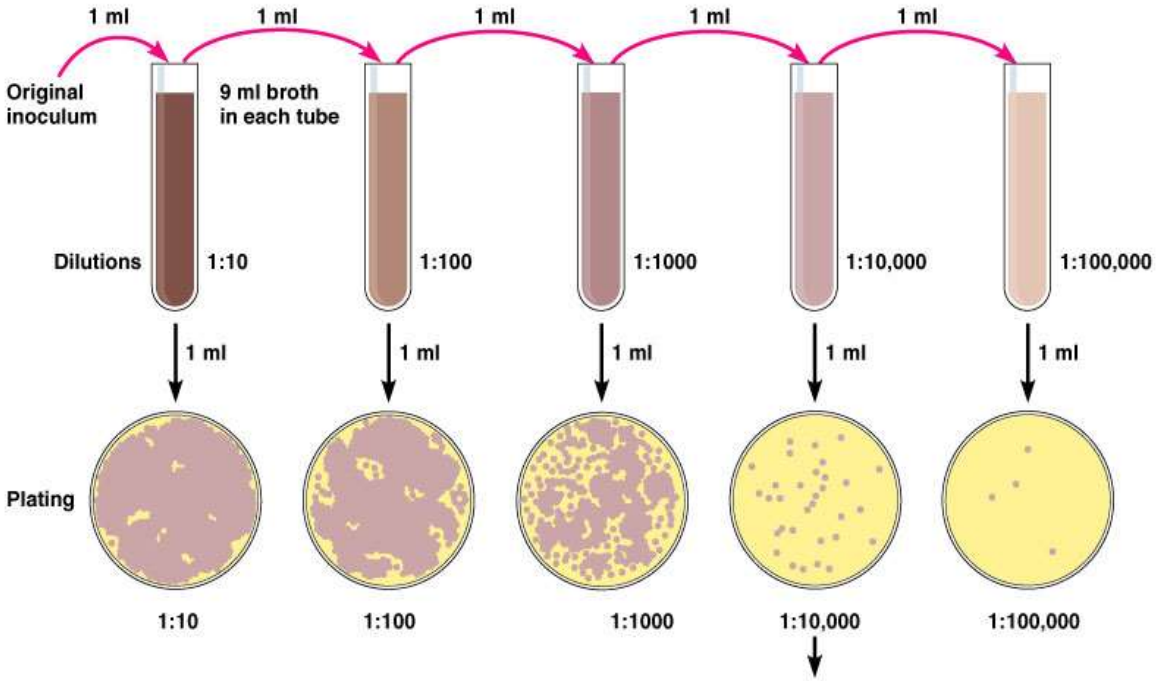
- **Lag Phase:** period of little or no cell division since cells do not reproduce immediately a new medium (1 hour to several days)
- **Log Phase or Exponential Growth Phase:** cells begin to divide, enter period of growth or logarithmic increase (straight line). Microorganisms are particularly sensitive to adverse conditions (radiation, antibiotics)
- **Stationary Phase:** growth rate slows, number of microbial deaths balances number of new cells. This is due to exhaustion of nutrients, changes of pH.
- **Death Phase:** logarithmic decline phase starts and continues until the population is diminished to a few cells or dies out completely



Direct Measurements of Microbial Growth:

1. Plate Count Method: Perform serial dilutions of a sample and plate each on an agar-medium plate

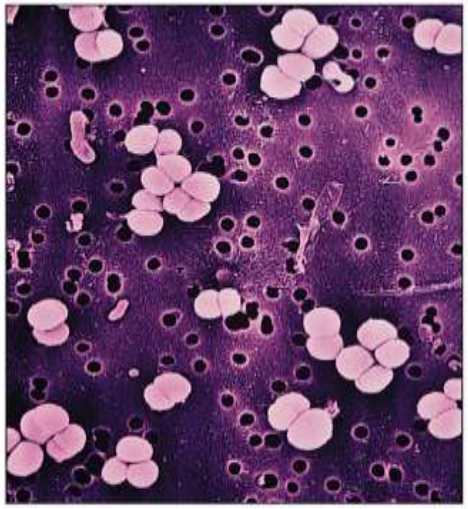
After incubation, count colonies on plates that have 25-250 colonies (CFUs)



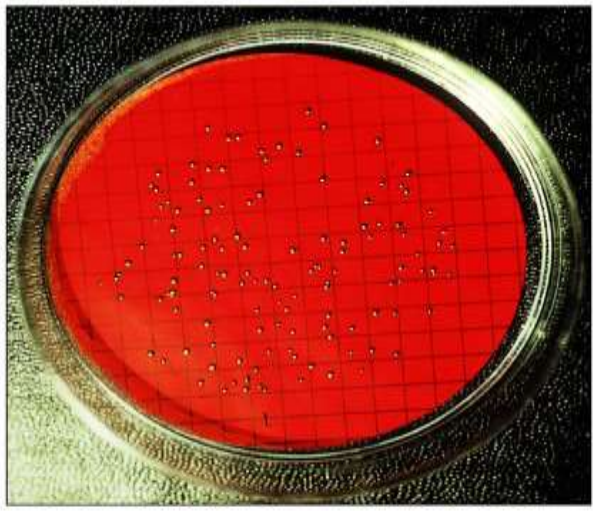
Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000/ml in sample.)

Direct Measurements of Microbial Growth:
2. Filtration

Bacteria are filtered out of a liquid and then transferred to a Petri dish



(a) The bacteria in 100 ml of water were sieved out onto the surface of a membrane filter.



(b) Such a filter as shown in photo (a) with the bacteria much more widely spaced, was placed on a pad saturated with liquid Endo medium, which is selective for gram-negative bacteria. The individual bacteria grew into visible colonies. One hundred twenty-four colonies are visible, so we would record 124 bacteria per 100 ml of water sample.

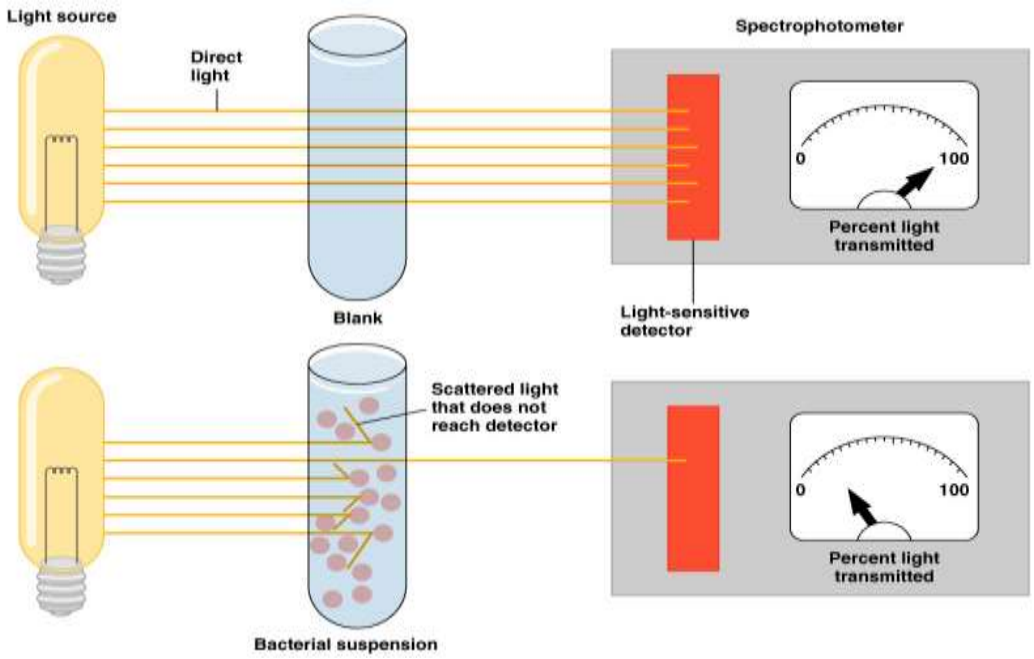
Direct Measurements of Microbial Growth:
3. Direct Microscopic Count

$$\text{Number of bacteria/ml} = \frac{\text{number of cells counted}}{\text{volume of area counted}}$$

$$\frac{14}{8 \times 10^{-7}} = 17{,}500{,}000$$

Estimating Bacterial Numbers
by Indirect Methods

Turbidity is not useful to measure contamination of liquids by relatively small numbers of bacteria



Estimating Bacterial Numbers by Indirect methods

- **Metabolic activity:** it assumes the amount of a certain metabolic product, such as acid or carbon dioxide, is in direct proportion to the number of bacteria present.
  - Determination of vitamin amount assay
- **Dry weight:** used to measure the growth of filamentous organisms.
  - Fungus is removed from growth medium, filtered, and dried in a desiccator and then weighed.

Aseptic technique that should be practiced

- Sterility of the solutions, the equipment's, the work place

TABLE 7.5 **Physical Methods Used to Control Microbial Growth**

Method	Mechanism of Action	Comment	Preferred Use
Heat			
1. Moist heat			
a. Boiling or flowing steam	Protein denaturation	Kills vegetative bacterial and fungal pathogens and almost all viruses within 10 min; less effective on endospores.	Dishes, basins, pitchers, various equipment
b. Autoclaving	Protein denaturation	Very effective method of sterilization; at about 15 psi of pressure (121°C), all vegetative cells and their endospores are killed in about 15 min.	Microbiological media, solutions, linens, utensils, dressings, equipment, and other items that can withstand temperature and pressure
2. Pasteurization	Protein denaturation	Heat treatment for milk (72°C for about 15 sec) that kills all pathogens and most nonpathogens.	Milk, cream, and certain alcoholic beverages (beer and wine)
3. Dry heat			
a. Direct flaming	Burning contaminants to ashes	Very effective method of sterilization.	Inoculating loops
b. Incineration	Burning to ashes	Very effective method of sterilization.	Paper cups, contaminated dressings, animal carcasses, bags, and wipes
c. Hot-air sterilization	Oxidation	Very effective method of sterilization, but requires temperature of 170°C for about 2 hr.	Empty glassware, instruments, needles, and glass syringes
Filtration	Separation of bacteria from suspending liquid	Removes microbes by passage of a liquid or gas through a screenlike material. Most filters in use consist of cellulose acetate or nitrocellulose.	Useful for sterilizing liquids (enzymes, vaccines) that are destroyed by heat

TABLE 7.8 Chemical Agents Used to Control Microbial Growth

Chemical Agent	Mechanism of Action	Preferred Use	Comment
Phenol and Phenolics			
1. Phenol	Disruption of plasma membrane, denaturation of enzymes	Rarely used, except as a standard of comparison.	Seldom used as a disinfectant or antiseptic because of its irritating qualities and disagreeable odor.
2. Phenolics	Disruption of plasma membrane, denaturation of enzymes	Environmental surfaces, instruments, skin surfaces, and mucous membranes.	Derivatives of phenol that are reactive even in the presence of organic material; O-phenylphenol is an example.
3. Bisphenols	Probably disruption of plasma membrane	Disinfectant hand soaps and skin lotions.	Triclosan is an especially common example of a bisphenol. Broad spectrum, but most effective against gram-positives.
Biguanides (Chlorhexidine)	Disruption of plasma membrane	Skin disinfection, especially for surgical scrubs.	Bactericidal to gram-positives and gram-negatives; nontoxic, persistent.
Halogens	Iodine inhibits protein function and is a strong oxidizing agent; chlorine forms the strong oxidizing agent hypochlorous acid, which alters cellular components.	Iodine is an effective antiseptic available as a tincture and an iodophor; chlorine gas is used to disinfect water; chlorine compounds are used to disinfect dairy equipment, eating utensils, household items, and glassware.	Iodine and chlorine may act alone or as components of inorganic and organic compounds.

TABLE 7.8	**Chemical Agents Used to Control Microbial Growth** (continued)		
Chemical Agent	**Mechanism of Action**	**Preferred Use**	**Comment**
Alcohols	Protein denaturation and lipid dissolution.	Thermometers and other instruments; in swabbing the skin with alcohol before an injection, most of the disinfecting action probably comes from a simple wiping away (degerming) of dirt and some microbes.	Bactericidal and fungicidal, but not effective against endospores or nonenveloped viruses; commonly used alcohols are ethanol and isopropanol.
Heavy Metals and Their Compounds	Denaturation of enzymes and other essential proteins.	Silver nitrate may be used to prevent gonorrheal ophthalmia neonatorum; mercurochrome disinfects skin and mucous membranes; copper sulfate is an algicide.	Heavy metals such as silver and mercury are biocidal.
Surface-Active Agents			
1. Soaps and acid-anionic detergents	Mechanical removal of microbes through scrubbing.	Skin degerming and removal of debris.	Many antibacterial soaps contain antimicrobials.
2. Acid-anionic detergents	Not certain; may involve enzyme inactivation or disruption.	Sanitizers in dairy and food-processing industries.	Wide spectrum of activity; nontoxic, noncorrosive, fast-acting.

Cell lysis

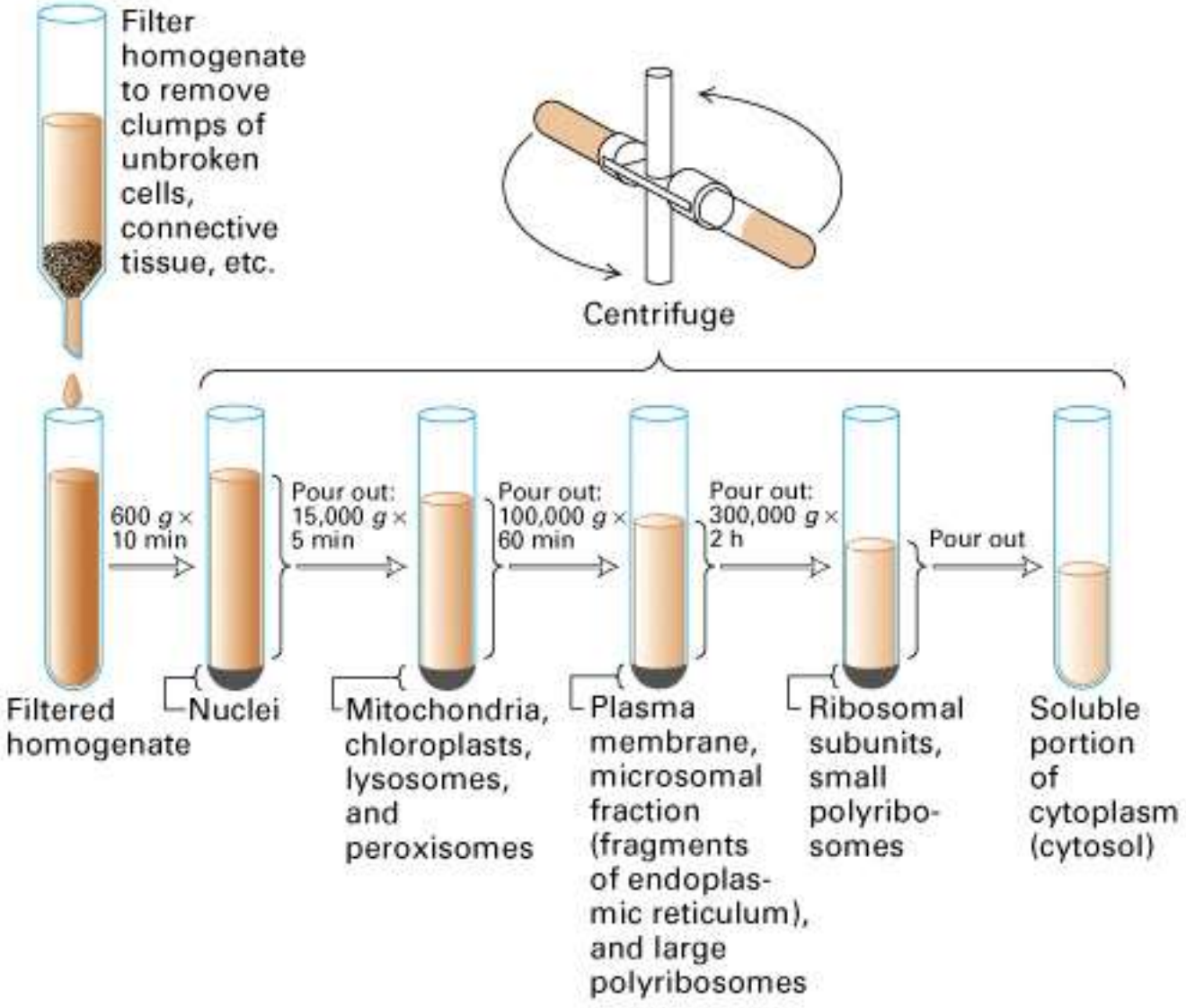
Cell lysis: rupture cell wall / plasma membrane,
--> release contents (organelles, proteins…)

1. Physical means (French Press)

2. Sonication

3. Osmotic shock

Differential Centrifugation



Protein purification – Column chromatography

-Protein mixture applied to column

-Solvent (buffer) applied to top, flowed through column

- Different proteins interact with matrix to different extents, flow at different rates

-Proteins collected separately in different fractions

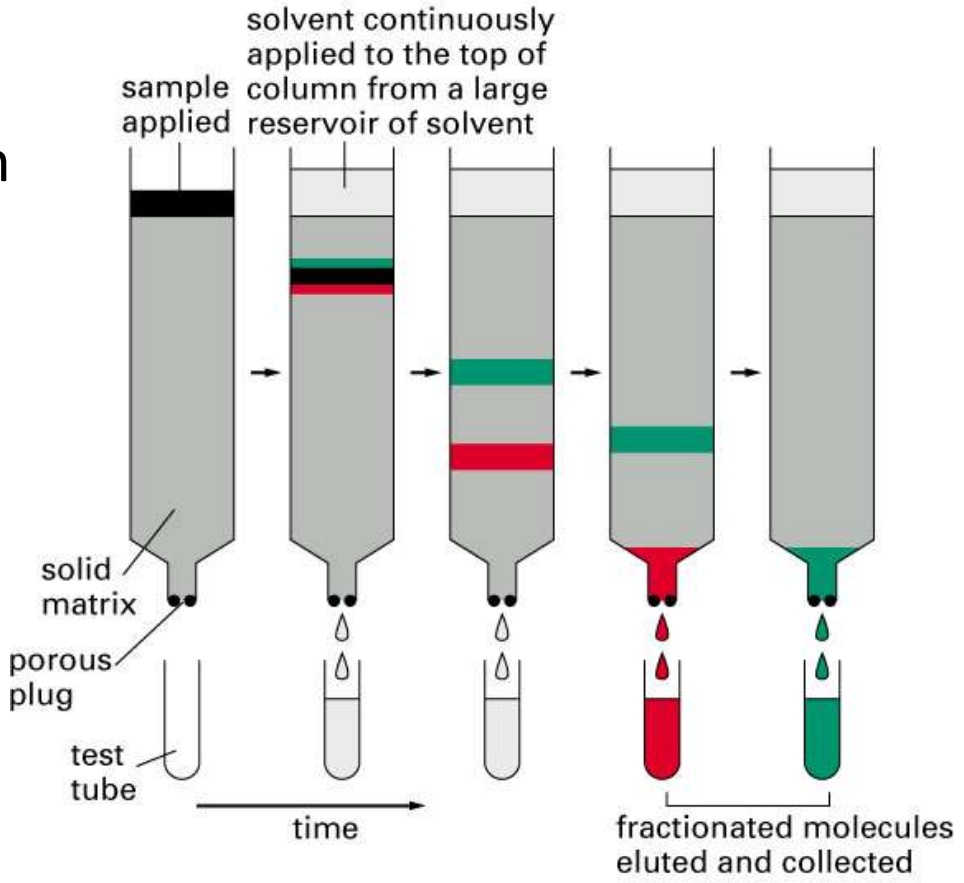


Figure 8–10. Molecular Biology of the Cell, 4th Edition.

Column Chromatography

Molecules can be separated on the basis of:

1. SIZE—Gel filtration

2. CHARGE—Ion exchange

3. SPECIFIC BINDING—Affinity

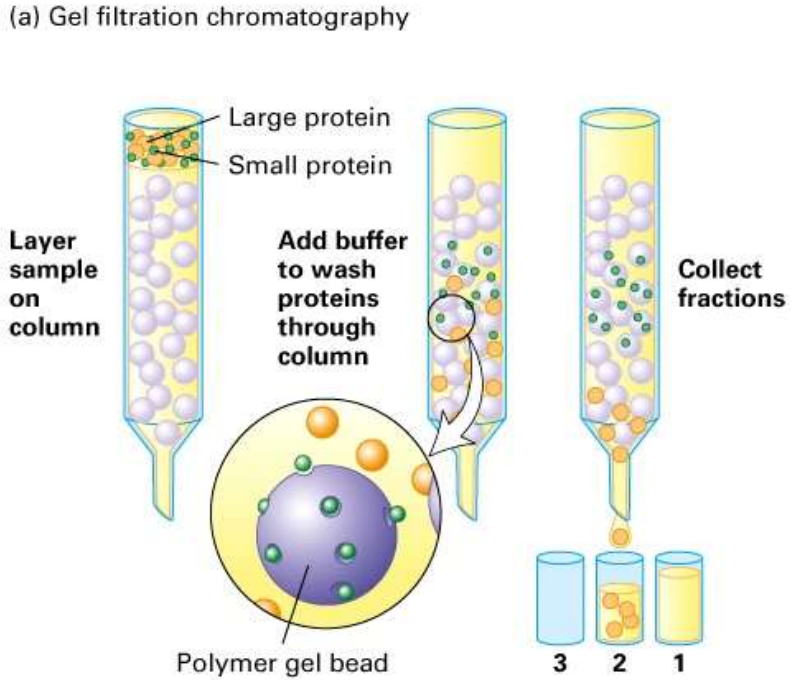
Gel filtration chromatography – Separation by size

Beads have different size pores

As column flows:

- large proteins excluded from pores and therefore flow rapidly

- small proteins enter pores and flow slowly



Ion exchange chromatography – separation by charge

Beads have charged group:

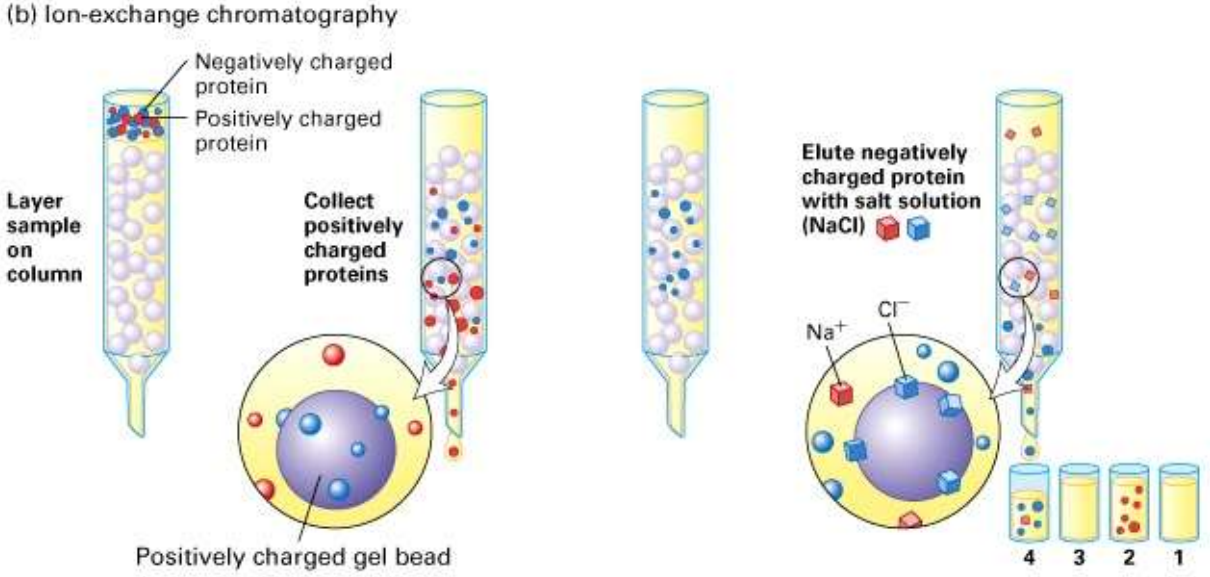
+ charge binds acidic amino acids

- charge binds basic amino acid

Different proteins bind with different affinity

Eluted with increasing amount of salt (NaCl or KCl)

Different proteins elute at different salt concentrations



Affinity chromatography
Separation by biological interactions

- Glutathione *S*-transferase (GST) is a 211 amino acid protein (26kDa) whose DNA sequence is frequently integrated into expression vectors for production of recombinant proteins. The result of expression from this vector is a GST-tagged fusion protein in which the functional GST protein (26kDa) is fused to the N-terminus of the recombinant protein.
- Because GST rapidly folds into a stable and highly soluble protein upon translation, inclusion of the GST tag often promotes greater expression and solubility of recombinant proteins than expression without the tag.
- GST-tagged fusion proteins also can be purified or detected based on the ability of GST (an enzyme) to bind its substrate, glutathione (GSH)

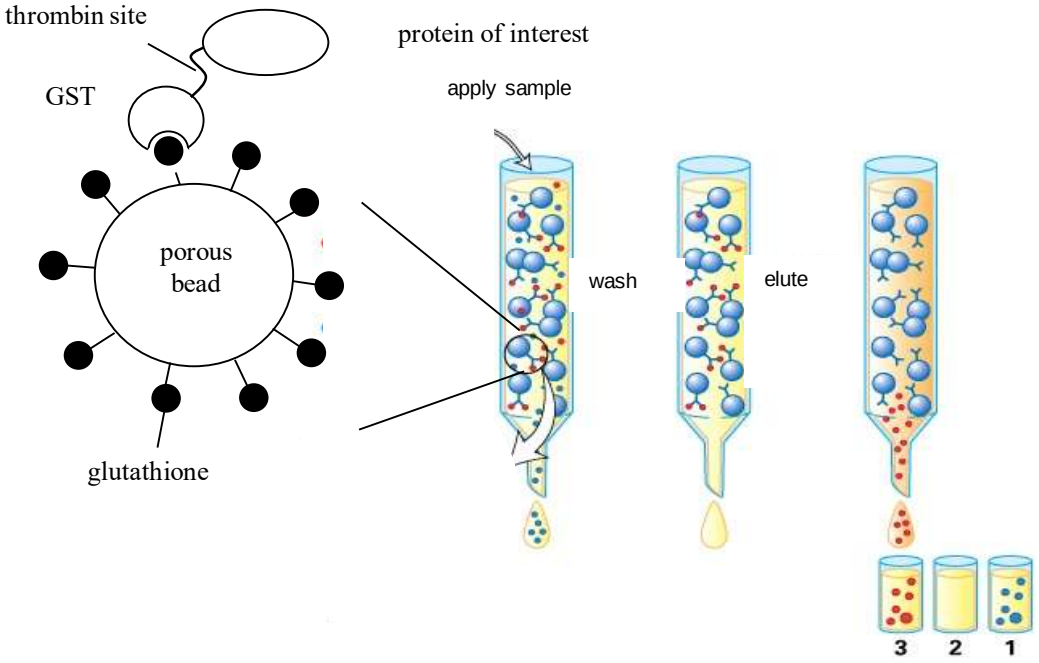
Affinity chromatography
Separation by biological interactions

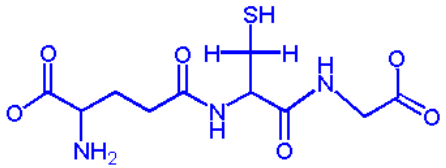
<u>Example: GST - Glutathione</u>

GST-tagged proteins bind to gluthatione on beads

Non-specifically or weakly bound proteins washed off

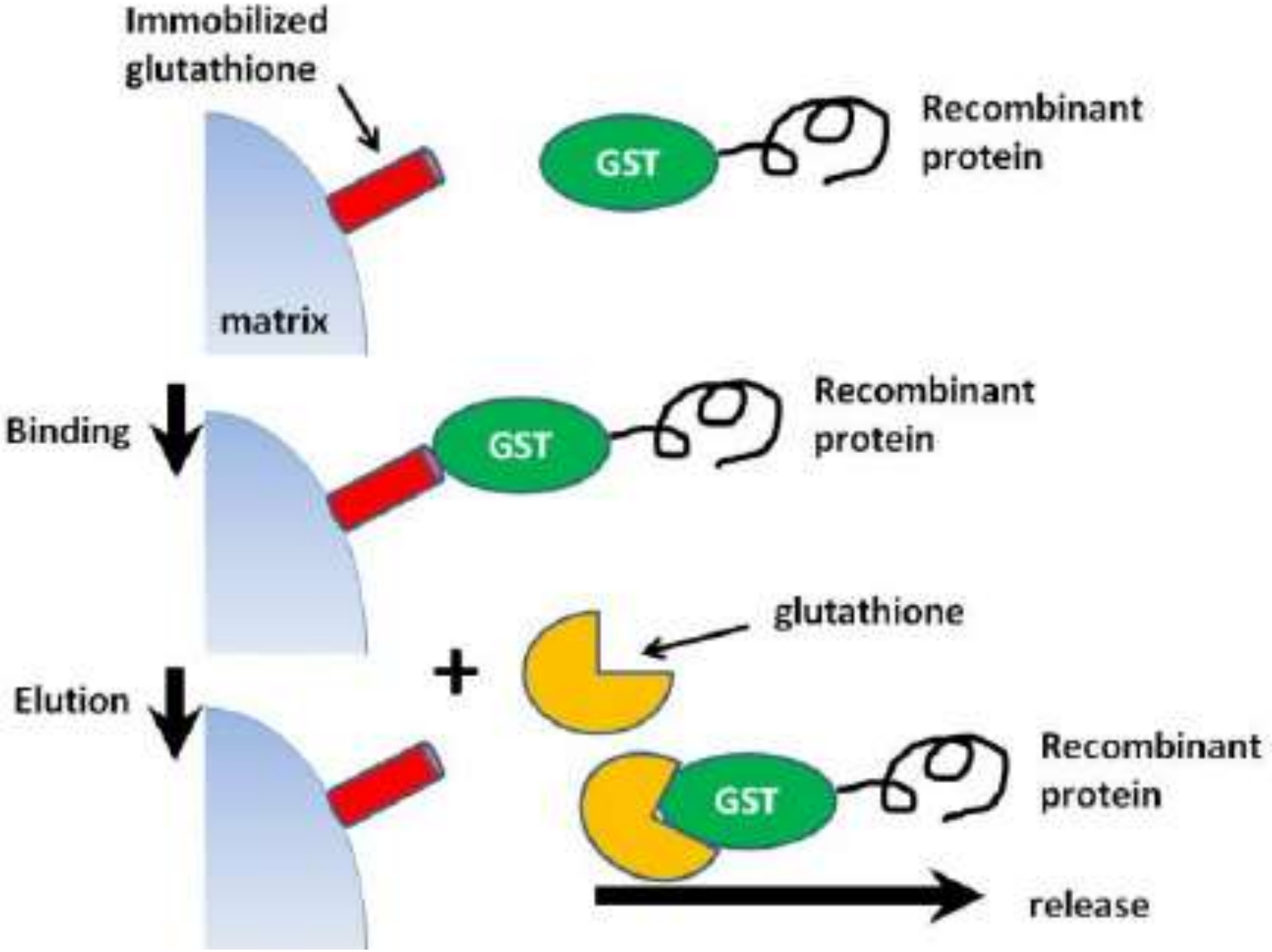
GST-tagged proteins eluted with glutathione (competitor) or thrombin (protease)





GLUTATHIONE (GSH)

gamma-glutamyl-cysteinyl-glycine


Immobilized
glutathione
matrix
GST
Recombinant
protein
Binding
GST
Recombinant
protein
glutathione
+
Elution
GST
Recombinant
protein
release

PolyHis tagged proteins

- The DNA sequence specifying a string of six to nine histidine residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6xHis tag fused to its N- or C-terminus.
- Expressed His-tagged proteins can be purified and detected easily because the string of histidine residues binds to several types of immobilized metal ions, including nickel, cobalt and copper, under specific buffer conditions. In addition, anti-His-tag antibodies are commercially available for use in assay methods involving His-tagged proteins.
- In either case, the tag provides a means of specifically purifying or detecting the recombinant protein without a protein-specific antibody or probe

Immobilized Metal Affinity Chromatography (IMAC)

- Supports such as beaded agarose or magnetic particles can be derivatized with chelating groups to immobilize the desired metal ions, which then function as ligands for binding and purification of biomolecules of interest.
- The chelators most commonly used as ligands for IMAC are nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA). Once IDA-agarose or NTA-agarose resin is prepared, it can be "loaded" with the desired divalent metal (e.g., Ni, Co, Cu, Fe). Using nickel as the example metal, the resulting affinity support is usually called Ni-chelate, Ni-IDA or Ni-NTA resin.
- Affinity purification of His-tagged fusion proteins is the most common application for metal-chelate supports in protein biology research. Nickel or cobalt metals immobilized by NTA-chelation chemistry are the systems of choice for this application
- The protein is eluted by high concentration of imidzole

START **N-term.** His-tag STOP

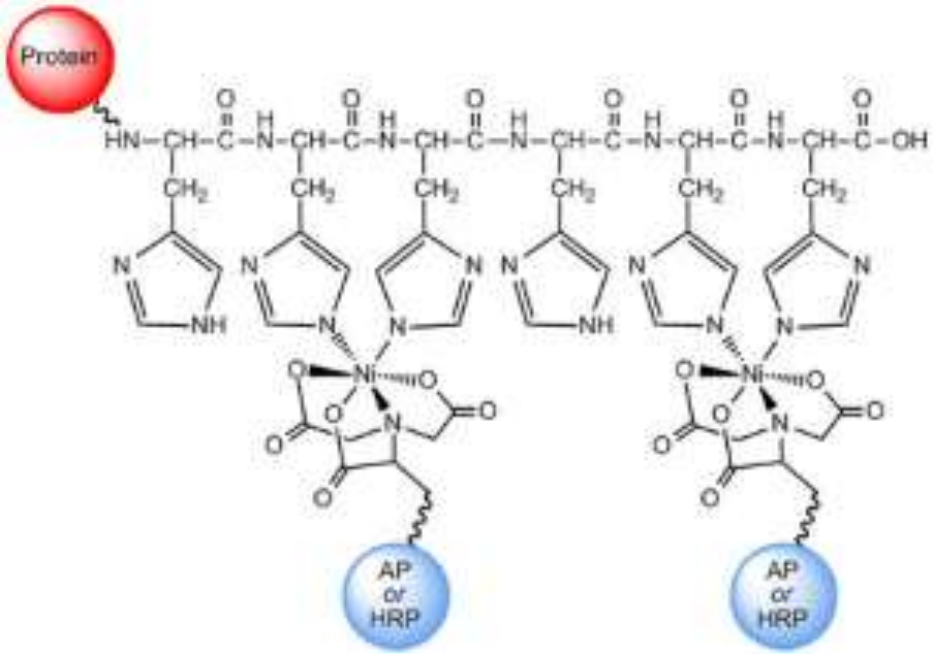
Kozak ↓ HIS HIS HIS HIS HIS HIS cDNA ↓

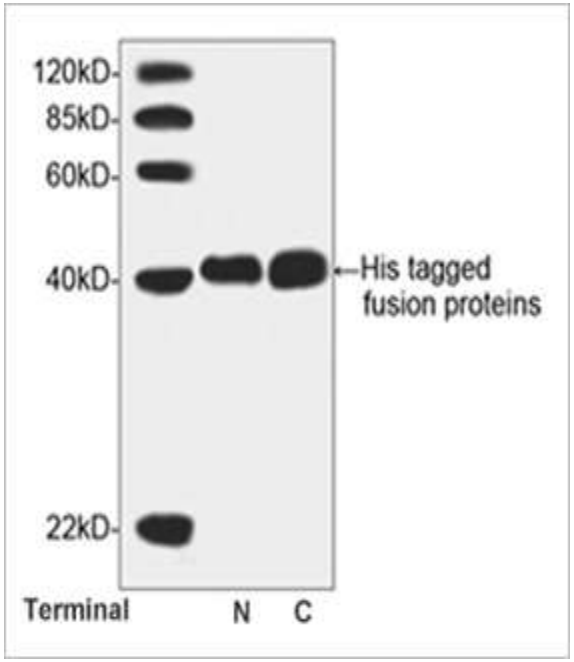
5' GCC ACC ATG cat cat cac cat cac cac NNN NNN NNN…TAG 3'

START **C-term.** His-tag STOP

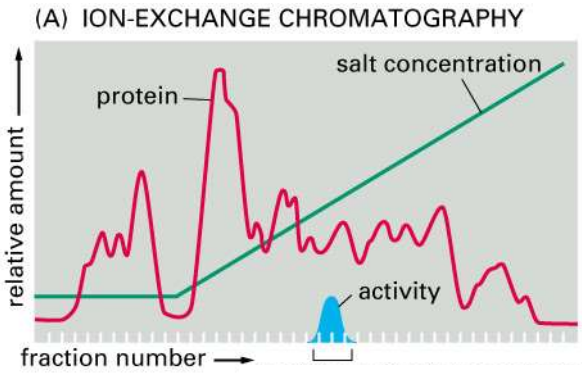
Kozak ↓ cDNA HIS HIS HIS HIS HIS HIS ↓

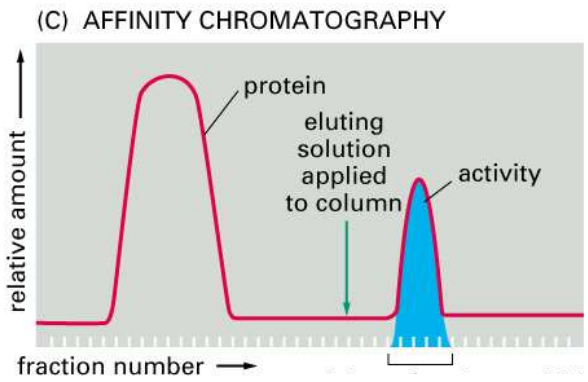
5' GCC ACC ATG NNN NNN NNN…cat cat cac cat cac cac TAG 3'





Protein purification by chromatography





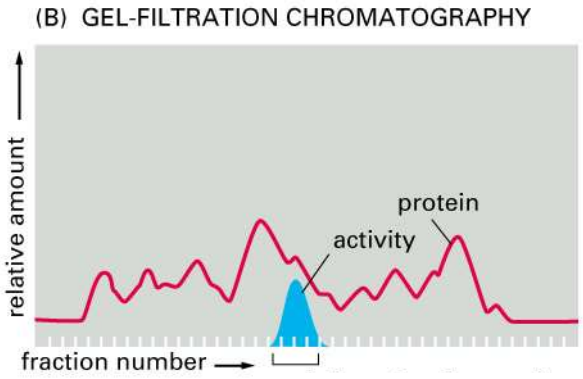


Figure 8–12 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Check the size and the purity of the protein
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. Heat sample with SDS and β -mercaptoethanol

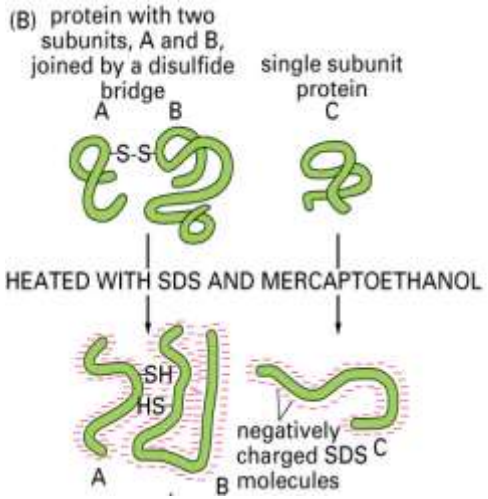
SDS = Detergent (anionic)
- Denatures proteins
- Coats proteins
- Each protein has similar mass/charge ratio

b-mercaptoethanol/DTT
- reduces disulfide bonds

2. Separate on polyacrylamide gel
- Protein migrates through gel matrix in electric field (from negative to the positive)

3. Use commassie blue or silver stain to visualize protein purity

SDS-PAGE



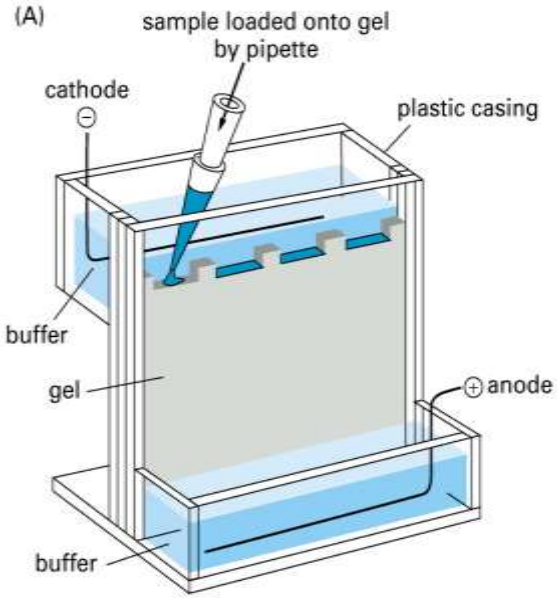


Figure 8–14 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Coomassie Blue/
Silver Staining