Protein Analysis

Enzyme-Linked Immunosorbent Assay (ELISA)

General Principles

- **Definition**: ELISA is an immunological assay used to detect and quantify proteins, hormones, or other molecules with high sensitivity and specificity.
- **Concept**: Based on the same antibody–antigen binding principle as immunoblotting, but:
    - Faster, More convenient, Highly sensitive (detection in the **nanogram [10^{-9} g] or lower range**).
- **Mechanism**: Uses enzyme-linked antibodies that catalyze reactions producing a detectable signal (commonly colorimetric).

Application Example: Pregnancy Testing

Biological Basis

- **Hormone detected**: Human Chorionic Gonadotropin (**hCG**).
    - Secreted by the **developing placenta** shortly after fertilization.
    - Essential in maintaining pregnancy (stimulates corpus luteum to produce progesterone).
    - Excreted in urine → allows for non-invasive testing.
- **Pregnant vs. Non-pregnant**:
    - Pregnant women: detectable hCG in urine.
    - Non-pregnant women: no hCG present.

Test Strip Structure

The commercial test strip is divided into three functional **zones**:

1. **Reaction Zone (R)**
    - Contains **enzyme-linked anti-hCG antibodies** (monoclonal, raised in mice).
    - These antibodies bind specifically to hCG if present in urine.
    - Complexes formed: **[enzyme-linked anti-hCG antibody + hCG]**.
    - If no hCG is present, antibodies remain unbound.
2. **Test Zone (T)**
    - Immobilized **anti-hCG antibodies** are fixed on the strip.
    - They capture the **hCG portion** of the antibody–antigen complexes from the reaction zone.
    - The enzyme linked to the first antibody reacts with dye molecules (substrates), producing a **colored band**.
    - Color at T zone = **Positive test (hCG present)**.
3. **Control Zone (C)**
    - Contains immobilized **anti-mouse antibodies**.
    - These bind to the **enzyme-linked anti-hCG antibodies** (whether or not they carry hCG).
    - Ensures the test is functional → should **always show a colored band** if the strip is working correctly.

Mechanism of Action

1. **Sample Application**: Urine is applied to strip and moves upwards via **capillary action**.
2. **Reaction Zone**
    - If hCG present → binds to enzyme-linked anti-hCG antibody.
    - Complex migrates further up the strip.
    - If no hCG → antibodies migrate unbound.
3. **Test Zone**
    - Complexes containing hCG are **captured by immobilized anti-hCG antibodies**.
    - Enzyme acts on dye substrate → **colored band forms**.
    - If no hCG present → migrating antibodies cannot be captured, no color forms here.
4. **Control Zone**
    - Unbound enzyme-linked antibodies (whether bound to hCG or not) are caught by anti-mouse antibodies.
    - Enzyme–substrate reaction produces a **colored band**.
    - Confirms strip functionality.

Interpretation of Results

- **Positive Test** - Two colored bands: **T zone (hCG present)**, **C zone (control)**
- **Negative Test** - One colored band: **C zone only** (no hCG present, but test functional).
- **Invalid Test** - No band in the **C zone** → strip malfunction or insufficient sample.

Protein Sequencing

Definition

- Protein sequencing: determination of the **linear order of amino acids** in a protein or peptide chain.
- Essential for:
    - Understanding protein structure and function.
    - Comparing homologous proteins across species.
    - Identifying mutations and variants.
    - Designing synthetic peptides or recombinant proteins.

Edman Degradation

Principle

- **Stepwise identification** of the **N-terminal residue** of a peptide.
- Achieved by repetitive cleavage and analysis of one residue at a time.
- Works best for **peptides < 50 residues** (larger proteins must be cleaved first).

Chemistry

1. **Labeling**:

- N-terminal residue reacts with **phenylisothiocyanate (PITC)** under mildly alkaline conditions.
- Forms a cyclic derivative: **phenylthiocarbamoyl (PTC) peptide**.

2. **Cleavage**:
   - Treated with **anhydrous acid** → releases the **N-terminal amino acid derivative** (phenylthiohydantoin, PTH-amino acid).
   - The rest of the peptide chain remains intact.
3. **Identification**: The liberated **PTH-amino acid** is identified via **chromatography** (ion-exchange or HPLC).
4. **Repetition**: The shortened peptide undergoes the same cycle until the sequence is determined.

Advantages

- Leaves the remaining peptide **intact**, allowing iterative sequencing.

Limitations

- Accuracy decreases after ~50 cycles due to **incomplete cleavage** and **residual carryover**.
- Large proteins require **prior cleavage into smaller peptides**.

Cleavage Methods for Large Proteins

Large proteins must be broken down into shorter peptides, which can then be sequenced and assembled into the complete protein sequence.

1. Chemical Cleavage

- **Reagent**: **Cyanogen Bromide (CNBr)**.
- **Specificity**: cleaves at the **C-terminal side of methionine (Met)** residues.
- Example: Protein with 10 Met residues → ~11 peptide fragments.
- Mechanism: CNBr converts Met residues into homoserine lactone, facilitating cleavage.

2. Enzymatic Cleavage (Endopeptidases)

- **Definition**: Enzymes that cleave peptide bonds **within the chain** at specific amino acid residues. This produces smaller peptides suitable for sequencing.

Enzyme	Cleavage Site (of target residue)	Cleaves After	Doesn't Cleave If Followed/Preceded By
Trypsin	C-terminal	Arg, Lys	Pro (after target)
Chymotrypsin	C-terminal	Phe, Tyr, Trp	Pro (after target)
Elastase	C-terminal	Ala, Gly, Ser, Val	Pro (after target)
Pepsin	**N-terminal**	Leu, Phe, Trp, Tyr	Pro (**before** target)

3. Exopeptidases

- **Definition**: Enzymes that sequentially remove amino acids from the **N-terminal (aminopeptidases)** or **C-terminal (carboxypeptidases)** of peptides.
- **Note**: Unlike Edman degradation, exopeptidases lack the same stepwise chromatographic identification precision and are less commonly used for full sequencing.

Protein Sequencing via DNA/RNA Analysis

- **Known gene sequence**:
  - Straightforward: translate codons → deduce amino acid sequence.
  - Tools: genetic code table ("codon table").
- **Unknown gene sequence (newly isolated protein)**:

1. Sequence a short peptide fragment by Edman degradation.
2. Deduce possible nucleotide codons for that fragment.
   - Due to degeneracy of the genetic code, multiple DNA sequences may code for the same peptide → often design multiple candidate probes.
3. Synthesize a **complementary DNA (cDNA) probe** to the mRNA of interest.
   - Probes are often labeled (radioactive or fluorescent).
4. Isolate **mRNA** from cells (important: ensures only expressed genes are captured; genomic DNA may include introns/noncoding regions).
5. Reverse-transcribe mRNA → **cDNA**.
6. Amplify cDNA via **PCR** for sequencing.
7. Sequence the DNA → deduce the full protein sequence.