Method of Enzyme Assay
Objective

- To study the different methods for determining enzyme activity.
- Use these method in diagnosis of certain diseases
How to follow a reaction?

**Enzyme assays**: Are laboratory methods for measuring enzymatic activity.

- Enzyme assays measure either the consumption of substrate or production of product over time.

\[ S \xrightarrow{E} P \]

- Different enzymes require different estimation methods depending on the type of reaction catalyzed, the nature of S and P or coenzyme.
Methods of quantitatively following enzyme reactions

How to follow an enzymatic reaction?
- First you must have complete knowledge about the reaction itself,

- Does the substrate or product has the ability to absorb light, fluorescence, any production of gases, production of H+?
- After that you can use this properties to detect the reaction
Methods of quantitatively following enzyme reactions

- **Fluorescence methods**
  - Using fluorometer

- **Manometric methods**
  - Using manometer

- **Electrode Methods**
  - Using a pH meter

- **Spectrophotometric methods**

- **Polarimetric Method**
  - Polarimeter
• In spectrophotometric assays, you follow the course of the reaction by measuring a change in how much light the assay solution absorbs.

**What is blank solution?**
It is a solution that contains everything except the compound to be measured.
Spectrophotometric methods

Sample

- absorb Invisible light (ultraviolet “UV”) 100 to 360 nm
  - Quartz cuvette

- absorb Visible range
  - Glass or plastic cuvette

[ If the light is in the visible region you can actually see a change in the color of the assay, these are called colorimetric assays ]
Examples of using Spectrophotometric methods

1- cases in which product absorb but not the substrate.
   e.g.

   ![Diagram](https://via.placeholder.com/150)

   Fumarate \(\rightarrow\) Fumarate hydratase \(\rightarrow\) malate

2- the Co-enzyme undergoes change in absorption upon reduction or oxidation

<table>
<thead>
<tr>
<th>Oxidized form</th>
<th>Reduced form</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>NADH</td>
</tr>
<tr>
<td>NADP</td>
<td>NADPH</td>
</tr>
</tbody>
</table>

If reduced form was product: increase the absorbance / min
If reduced form was substrate: decrease the absorbance / min
Enzyme assays can be split into two types:

- **Continuous assays,**
  where the assay gives a *continuous reading* of activity.

- **Discontinuous (Endpoint) assays,**
  Where the reaction is *stopped* and then the concentration of substrates/products determined.
If Substrate and Product cannot absorb light? What is the solution?

Example:

\[
\text{glutamate} + \text{pyruvate} \xrightleftharpoons[\text{ALT}]{\text{alanine}} \rightarrow \text{\alpha}\text{-ketoglutarate}
\]

It is also called: Serum glutamic pyruvic transaminase (SGPT) or Alanine aminotransferase (ALT).
Alanine transaminase (ALT) in serum

- Continues assay
- Discountouse assay
Alanine transaminase (ALT)

- ALT is an enzyme that catalyzes a type of reaction (transamination) between an amino acid and α-keto acid.
- It is important in the production of various amino acids.

**transamination reaction** involves removing the amino group from the amino acid, leaving behind an α-keto acid, and transferring it to the reactant α-keto acid and converting it into an amino acid.

It is also called: Serum glutamic pyruvic transaminase (SGPT) or Alanine aminotransferase (ALT).
ALT diagnostic importance

• ALT is found in serum (at low level) but is most commonly found in liver.

• thus, an elevated level ALT is a sensitive index of acute hepatocellular injury.

• Elevated serum ALT level are found in hepatitis, cirrhosis, and obstructive jaundice.

• NORMAL RANGE OF ALT:

  ( up to 42 ) U/L → males   ( up to 32 ) U/L → females
1-Continuous Assay

- The reading will be continues (1 min for 3 min).
- Because nether the S nor P can absorb light, the following can be done: First we will add the enzyme to the Substrate

1. Alanine + α-ketoglutarate → Pyruvate + glutamate
2. Another enzyme (LDH) and NADH+H+ will be added:
3. Pyruvate + NADH+H+ → L-Lactate+ NAD+ +H₂O

The absorbance at 340nm is measured each minute without stopping the reaction, resulting in decreased readings due to the oxidation of NADH.
Principle

\[
\text{Alanine} + \alpha\text{-ketogulutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{glutamate} \\
\text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{NAD}^+ \\
\text{Lactate} + \text{H}_2\text{O}
\]

The absorbance at 340nm is measured each minute, resulting in decreased readings due to the oxidation of NADH.
Method

Pipette into clean and dry test tubes:

<table>
<thead>
<tr>
<th>ALT Reagent</th>
<th>3 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-warm at 37° C for 3 minutes and add</td>
<td></td>
</tr>
<tr>
<td>Serum Sample</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Mix and incubated at 37 °C for 1 minute, then read absorbance (at 340 nm against distilled water) every minute for 3 minutes) and determine ΔA/min</td>
<td></td>
</tr>
</tbody>
</table>

Choose the following on the spectrophotometer:

2) Applications → 2) Simple Kinetics → wave length (340 nm) → Seconds → Duration (180 sec = 3 min) → Intervals (60 sec= 1 min) → Print Data Table (off) → Press start (2 times)
## Results

<table>
<thead>
<tr>
<th>Time</th>
<th>Absorbance 340nm</th>
<th>$\Delta A/\text{min} = \frac{(A1-A2)+(A2-A3)}{2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>A1</td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>A2</td>
<td></td>
</tr>
<tr>
<td>3 min</td>
<td>A3</td>
<td></td>
</tr>
</tbody>
</table>

## Calculations

ALT Activity (U/L) = $\Delta A/\text{min} \times 1768$

ALT Activity (U/L) =
Discontinuous Assay

• In this method **ALT** catalyzes the following reaction

  Alanine + α-ketoglutarate \( \rightarrow \) pyruvate + glutamate

• ALT is assayed by following formation of **pyruvate**.

• The addition of acidic 2,4-dinitrophenylhydrazine (DNPH) lead to the formation of 2,4-dinitrophenylhydrazone, then NaOH will be added. So that it may be measured at 546nm.
This assay as an example of colorimetric endpoint assay
Why colorimetric? Because it will give a brown color
Normal Range: 10-40 units per liter (U/L)
Method:

<table>
<thead>
<tr>
<th></th>
<th>BLANK</th>
<th>SAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT Reagent</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Pre-warm at 37 °C for 5 minutes and add:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Serum Sample</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Mix, and incubate at 37 °C for exactly 20 minutes, and add:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color Reagent (DNPH)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Mix, and return at 37 °C for exactly 10 minutes, then add:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color Developer (NaOH)</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Mix, and return to 37 °C for exactly 5 minutes. Read absorbance of all tubes at 546nm against blank.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Precautions

- COLOR REAGENT contains 1 N Hydrochloric acid which causes burns.
- COLOR DEVELOPER contains 0.5 N Sodium hydroxide which is corrosive.

In case of contact, flush affected area with large amounts of water. Seek medical attention.
Results:

<table>
<thead>
<tr>
<th>Absorbance at 546 nm</th>
<th>ALT activity (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>2.5</td>
</tr>
<tr>
<td>0.050</td>
<td>5.5</td>
</tr>
<tr>
<td>0.075</td>
<td>9</td>
</tr>
<tr>
<td>0.100</td>
<td>12</td>
</tr>
<tr>
<td>0.125</td>
<td>17</td>
</tr>
<tr>
<td>0.150</td>
<td>21</td>
</tr>
<tr>
<td>0.175</td>
<td>25</td>
</tr>
<tr>
<td>0.2</td>
<td>30</td>
</tr>
<tr>
<td>0.225</td>
<td>35</td>
</tr>
<tr>
<td>0.250</td>
<td>41</td>
</tr>
</tbody>
</table>

- The data shown in the table is used to convert absorbance at 546 nm into enzymatic activity in U/L of serum.

- Draw graph using the data in table with absorbance on the Y-axis and enzymatic activity in U/L on the X-axis.

**Note:** Don’t forget title of the graph “Standard Curve” and the x-axis and y-axis with their units.

-Absorbance at 546 nm = ........

-ALT (SGPT) activity (from graph)= ...........U/L
Discussion

- Mention the diagnostic importance of ALT

- Explain the difference in the principle of each ALT assay.

- Compare your result with ALT normal range [in males], and diagnose the patient's state (what disease could the patient have or not).