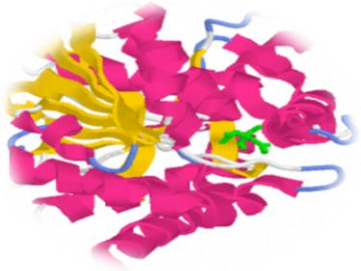
322 BCH

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.

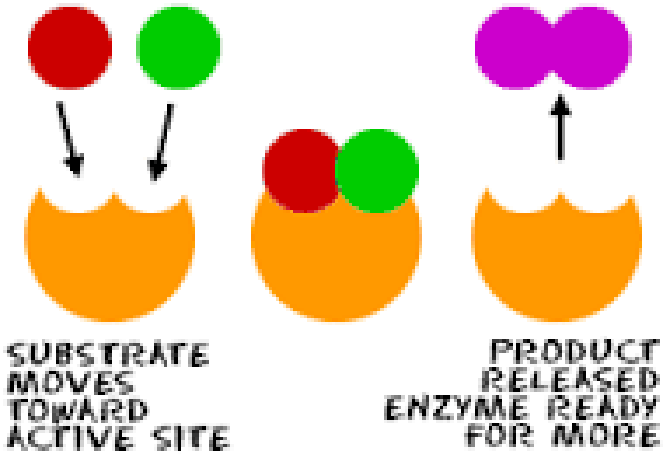
- **Different enzymes require different estimation methods depending on the type of reaction catalyzed, the nature of S and P or coenzyme.**

Methods of quantitatively following enzyme reactions

How to follow an enzymatic reaction?

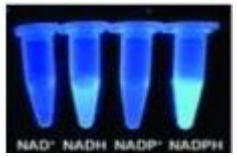
- First you must have complete knowledge about the reaction itself,

- 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



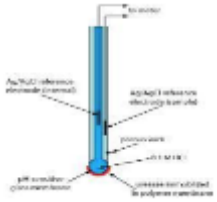
Methods of quantitatively following enzyme reactions

Fluorescence methods
Using fluorometer



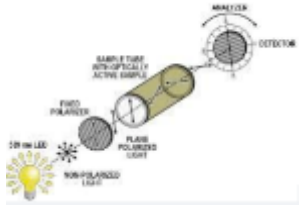
Manometric methods
Using manometer.

Eletrode Methods
Using a pH meter



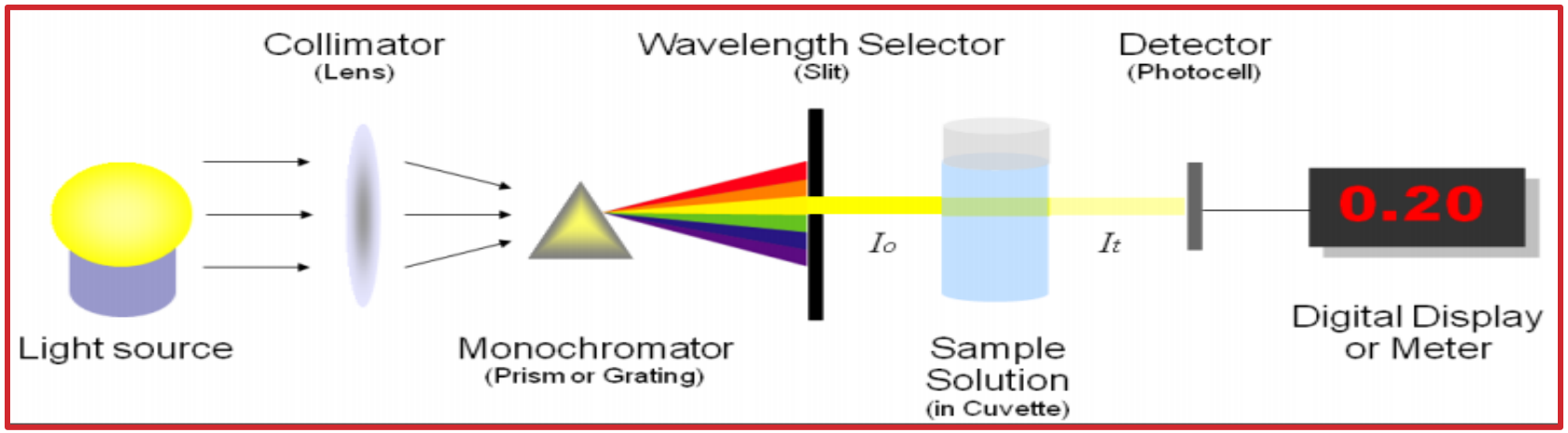
Spectrophotometric methods

Polarimetric Method
polarimeter



Spectrophotometric methods

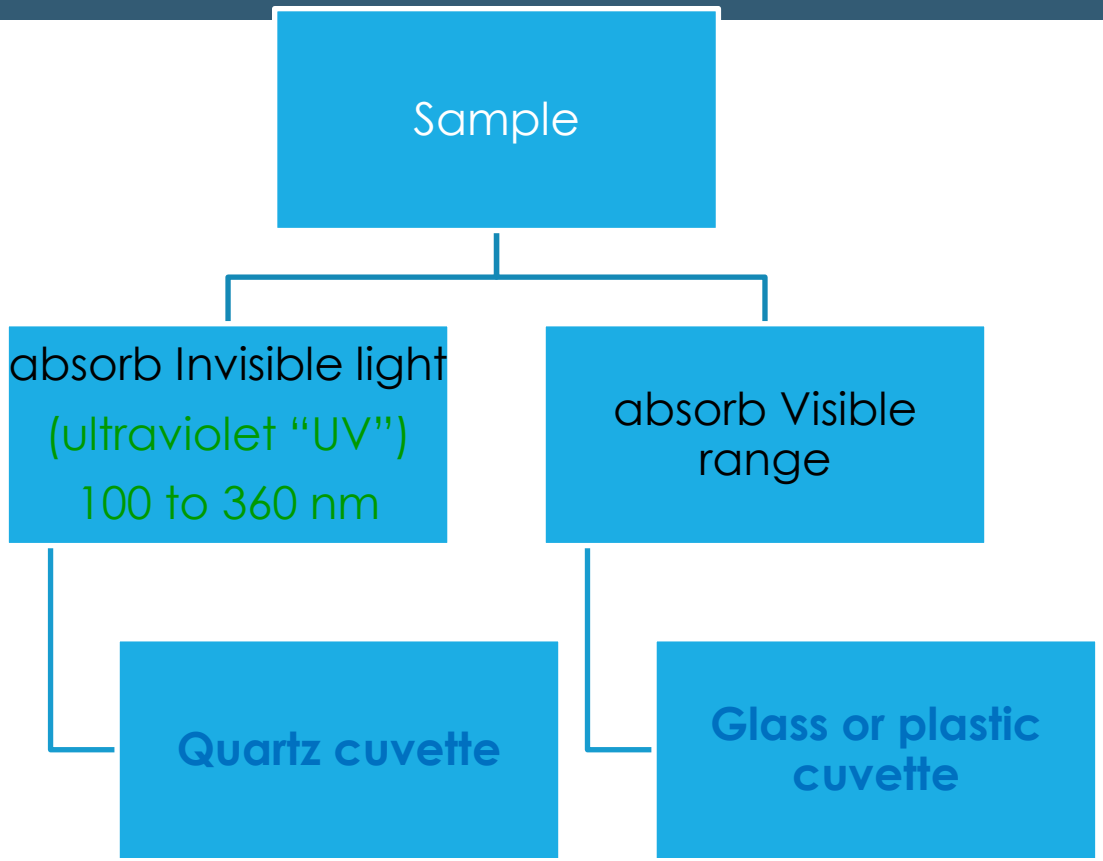
- In spectrophotometric assays, you follow the course of the reaction **by measuring a change in how much light the assay solution absorbs.**



What is blank solution?

It is a solution that contains everything except the compound to be measured.

Spectrophotometric methods



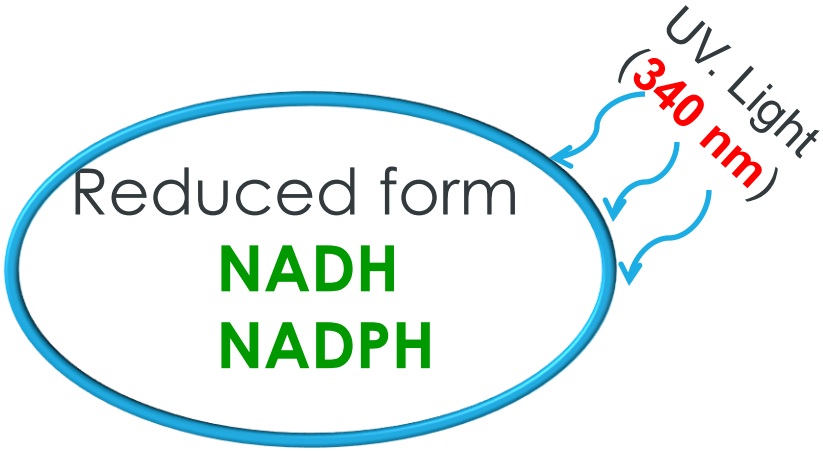
[If the light is in the visible region you can actually see a change in the color of the assay, these are called **colorimetric assays**]

Examples of using Spectrophotometric methods

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

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

Oxidized form
NAD
NADP



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**,

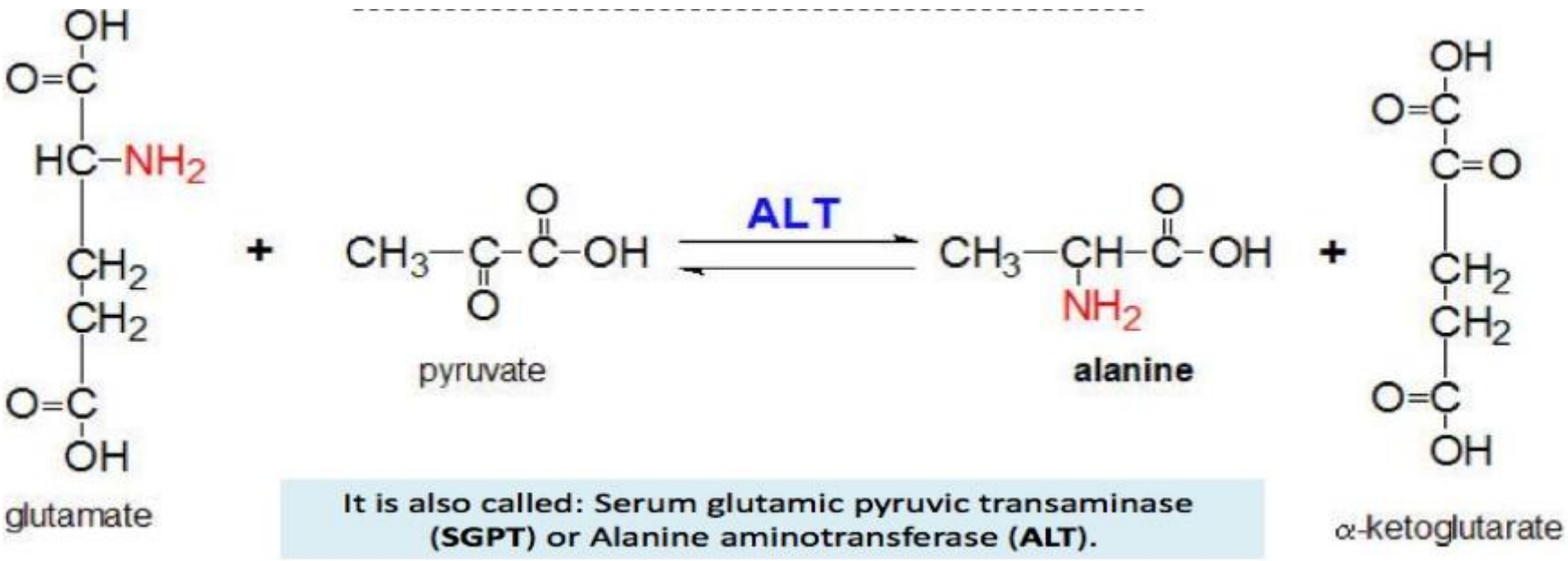
where the assay gives a continuous reading of activity.

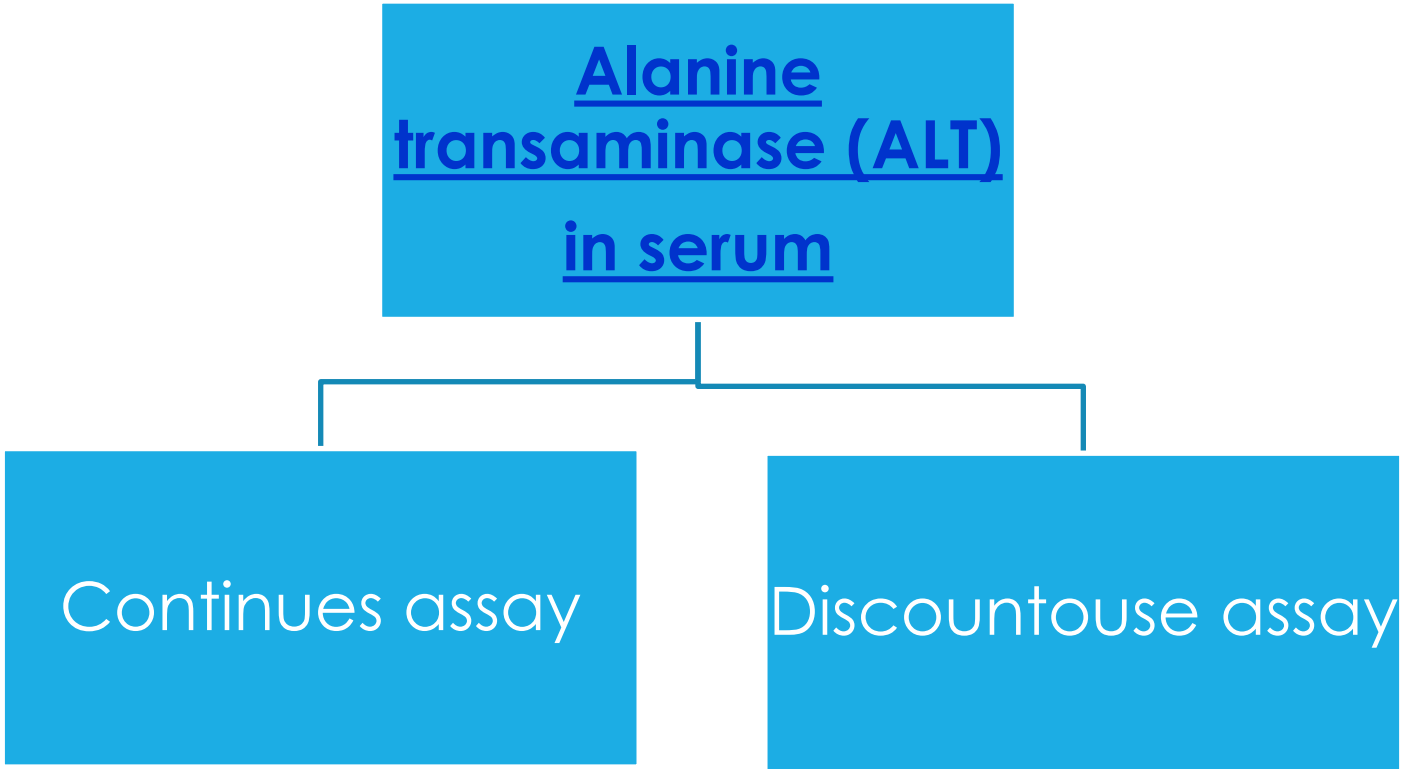
- **Discontinuous (Endpoint) assays**,

Where the reaction is stopped and then the concentration of substrates/products determined.

If Substrate and Product can not absorb light? What is the solution?

- Example:

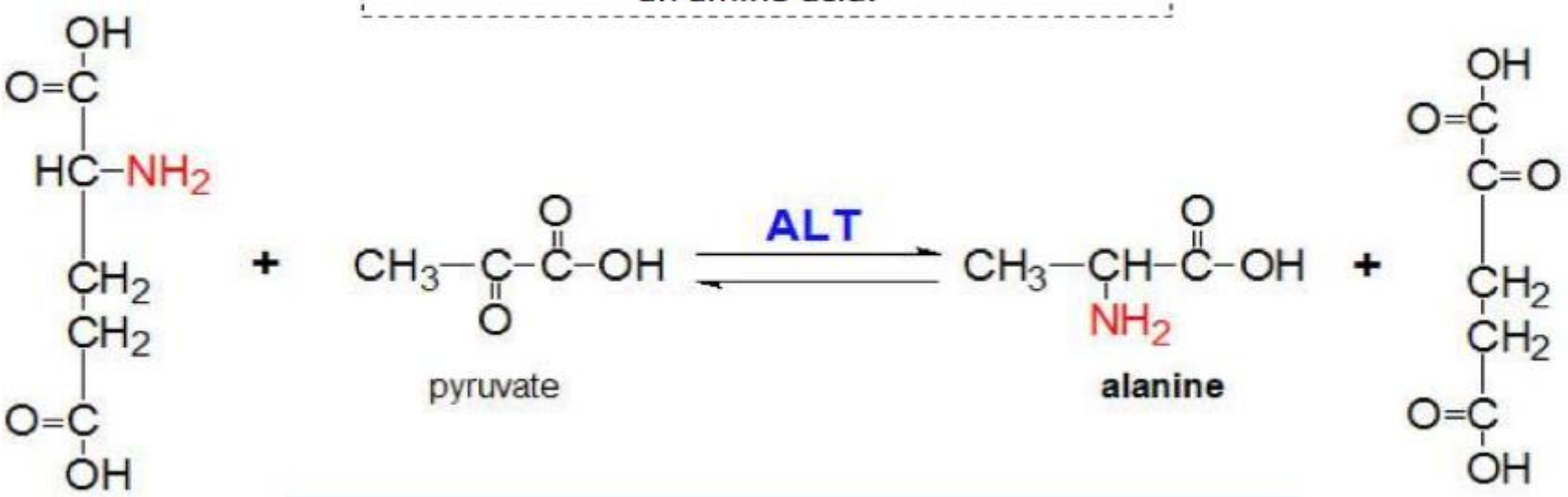



Alanine transaminase (ALT) in serum
Continues assay
Discountouse assay

Alanine transaminase (ALT)

- ALT is an enzyme that catalyzes a type of reaction (transamination) between an amino acid and a-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 is 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

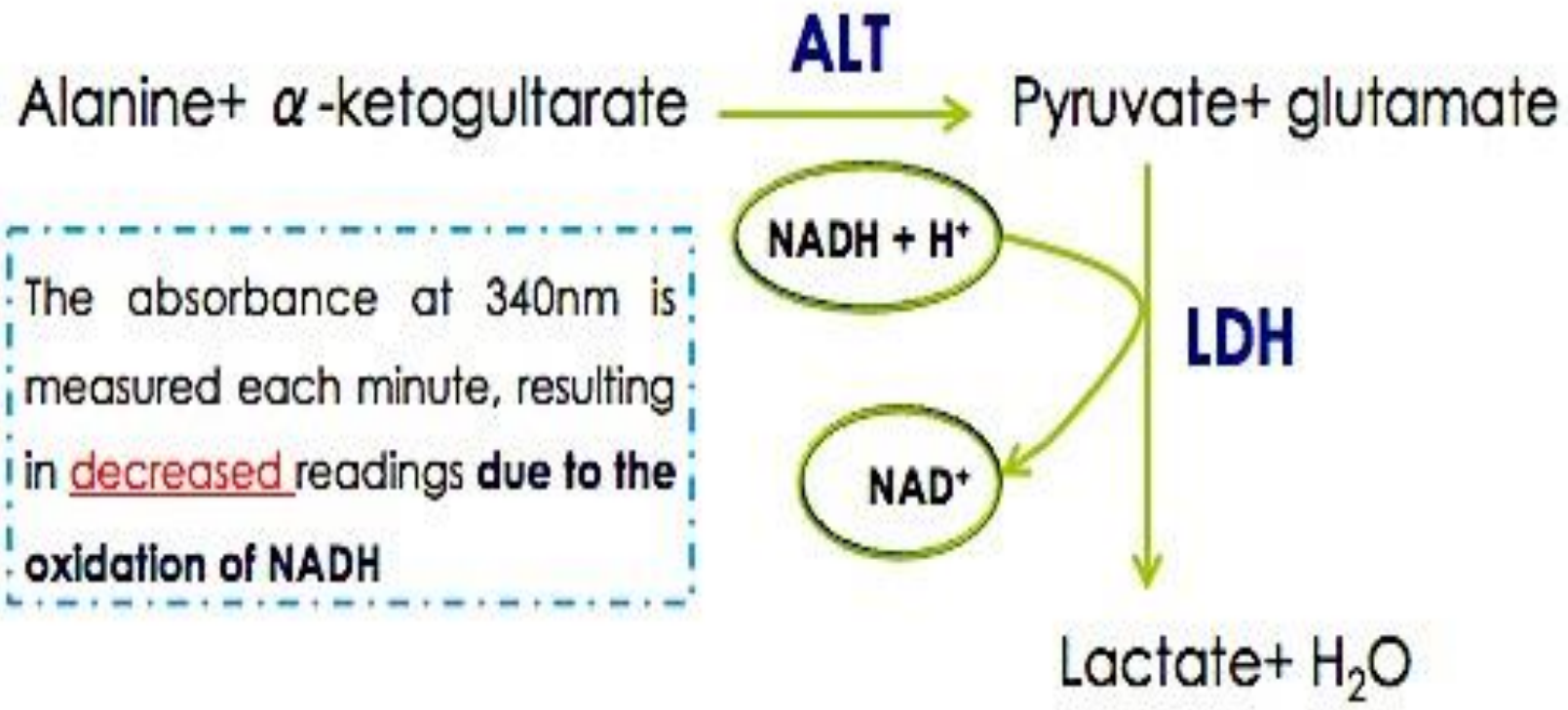
1-Continuous Assay

- 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^+ +H_2O**

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

Principle



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:

ALT Reagent	3 ml
Pre-warm at 37° C for 3 minutes and add	
Serum Sample	0.2 ml
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	

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

Time	Absorbance 340nm		ΔA/min=((A1-A2)+(A2-A3))/2
1 min	A1		
2 min	A2		
3 min	A3		

Calculations

ALT Activity (U/L) = ΔA/min x 1768

ALT Activity (U/L) =

Discontinuous Assay

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

  Alanine + a-ketoglutarate → 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.

Reaction-discountinouse

Alanine + a-ketoglutarate → pyruvate + glutamate

2,4-dinitrophenylhydrazine

2,4-dinitrophenylhydrazone

- **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:

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

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:

Absorbance at 546 nm	ALT activity (U/L)
0.025	2.5
0.050	5.5
0.075	9
0.100	12
0.125	17
0.150	21
0.175	25
0.2	30
0.225	35
0.250	41

- 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).