M.Sc., Biochemistry **2018-2020**

Semester III

18BCP311 **PRACTICAL – V** **4H-2C**

CLINICAL ENZYMES AND IMMUNOLOGY

Instruction hours/week: L:0 T:0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

Course objectives

- To understand the principles and diagnostic importance of various clinically important enzymes
- To determine the activity of various clinically important enzymes
- To learn the immunological experiments and understand the antigen – antibody reactions.
- To analyse a case for various diseases like diabetes, cardiac diseases and cancer.

Course outcomes (CO's)

After learning this practicals the students could be able

1. to analyse the biological samples and can be able to interpret the results
2. By doing a case study they will be getting a clear picture of various diseases and their etiology.

ENZYMOLOGY

1. Determination of the activity of the following serum enzymes:
    a. LDH
    b. Acid phosphatase
    c. Alkaline phosphatase
    d. Aspartate amino transferase
    e. Alanine amino transferase
    f. 5' nucleotidase
    g. Sodium potassium ATPase
    h. Ceruloplasmin

IMMUNOLOGY (DEMONSTRATION)

2. Raising of antibodies- single soluble and particulate antigen
3. Immunodiffusion- single radial and double diffusion.
4. Immunoelectrophoresis.
5. Rocket immunoelectrophoresis
6. ELISA
7. Bacterial Agglutination: WIDAL
8. Antibody titration – ELISA

Case study-Report

9. Serum enzyme in liver disease
10. Serum enzyme in cardiac disease
11. Serum enzyme in cancer disease

12. Glucose Tolerance Test

SUGGESTED READINGS

1. Jayaraman, J., (2007). Laboratory Manual in Biochemistry, New Age International Publishers New Delhi.

2. Sadasivam, S., and Manickam, A., (2009). Biochemical Methods, New Age International Publishers, New Delhi.

3. Singh, S.P., (2009). Practical Manual of Biochemistry, CBS Publishers, New Delhi.

4. Talib, V. H., (2003). A Handbook of Medical Laboratory Technology, CBS Publishers, New Delhi.

5. David Wild, (2013). Elsevier; Immuno Assay Hand Book

KARPAGAM ACADEMY OF HIGHER EDUCATION

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Coimbatore – 641 021.

LECTURE PLAN
DEPARTMENT OF BIOCHEMISTRY

STAFF NAME: Dr. M. Sridhar Muthusami
SUBJECT NAME: CLINICAL ENZYMES AND IMMUNOLOGY - PRACTICAL
SUB.CODE:18BCP311
SEMESTER: III CLASS: II M.Sc., (BC)

S.NO	NAME OF THE EXPERIMENT	SUPPORT MATERIALS
1.	Determination of the activity of LDH in serum	T1: 461 - 465
2.	Determination of the activity of alanine transaminase in serum	T1: 293 – 295 T2: 113 - 116
3.	Determination of the activity of aspartate transaminase in serum	T1: 293 – 295 T2: 113 - 116
4.	Determination of the activity of acid phosphatase in serum	T1: 453 – 455
5.	Determination of the activity of alkaline phosphatase in serum	T1: 522 - 525
6.	Determination of the activity of 5’ nucleotidase in serum	T2: 449 - 504
7.	Determination of the activity of sodium potassium ATPase in serum	T1: 470 - 475
8.	Determination of the activity of ceruloplasmin in serum	T2: 560 - 565
9.	Raising of antibodies – Single soluble and particulate antigen	W1: www.getlifesciences.com
10	Immuno diffusion – Single radial and double diffusion	W2: www.himedialabs.com
11	Immuno electrophoresis – Rocket immune electrophoresis	W3&W4: www.himedialabs.com
12	ELISA	W5: www.himedialabs.com
13	Case study reports: Serum enzymes in liver diseases	
14	Case study reports: Serum enzymes in cardiac diseases	

15	Case study reports: Serum enzymes in cancer diseases	
16	Case study reports: Glucose tolerance test	

SUGGESTED READINGS:

T1: H. Varley (1988). Practical clinical Biochemistry; 4th edition

T2: Sadasivam S and Manickam A. 2009. Biochemical methods, New age International publishers, New delhi.

W1: www.getlifesciences.com

W2: www.himedialabs.com

W3: www.himedialabs.com

W4: www.himedialabs.com

W5: www.himedialabs.com

ENZYMOLOGY

Experiment. 1

Determination of the activity of the following serum enzymes:

a. ASSAY THE ACTIVITY OF SERUM LACTATE DEHYDROGENASE

AIM:

To assay the activity of serum lactate dehydrogenase in serum.

PRINCIPLE:

Lactate is converted to pyruvate which gives a brown color with 2,4 dinitrophenyl hydrazine which is measured at 440nm.

REAGENTS:

1. Glycine buffer

7.05gm of glycine and 5.85gm of Nacl are dissolved in distilled water and made up to1 liter.

2. Buffered substrate

125ml of glycine buffer and 75ml of 0.1N NaoH is added to 4.0 gm of lithium lactate.

3. Solution of NAD

10mg of NAD was dissolved in 2.0 ml of distilled water and kept at 4^0C /NAD retained its stability longer if dissolved in 0.2M nicotinamide.

4. 2, 4 – dinitrophenyl hydrazine

200mg of 2,4- dinitrophenyl hydrazine was dissolved in 1N Hcl and made up to 1litre with the same.

5. 0.4N NaoH

6. Standard sodium pyruvate

22mg of sodium pyruvate is dissolved in 100ml of buffered substrate.

Concentration = 2.0μm/ml, stock is directly used for experiement.

PROCEDURE:

1.0 ml of buffered substrate was pipetted out into two test tubes named, control and test, 0.2ml of serum was added to each of the test tubes followed by 0.2ml of H_2O to control alone.

The tubes were incubated at 37°C for 15minutes. At the end of incubation 0.2ml of NAD solution was added to the test (T) tube. Simultaneously a set of standard containing sodium pyruvate was run by taking 0.2 – 1.0ml of stock standard solution corresponding to concentration 0.4 – 2.0µm and volume was made up to 1.0ml with distilled water. Added 1.0ml of DNPH to all tubes including test control and incubated at 37°C for 15minutes. After incubation added 10ml of NaoH to all the tubes and read the brown color developed at 440nm after 15minutes.

From the stand graph activity of enzymes can be calculated by the amount of pyruvate liberated per minute.

Normal value = 17 – 240IU/L

RESULT:

The amount of pyruvate liberated per minute was found to be ----------

CALCULATION

Colorimeter reading ___ corresponds to ____ µm of pyruvate

That is 0.2 ml of serum contains ___ µm of pyruvate

Therefore 100 ml of serum liberate $\frac{0.1 \times 1000}{0.2}$

= ____ µm of pyruvate

Amount of pyruvate liberated is given by = ----------

= _____ IU/litre

REAGENTS	**B**	**S1**	**S2**	**S3**	**S4**	**S5**	**C**	**T1**	**T2**
Volume of working standard (ml)	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**-**	**-**	**-**
Concentration of working standard (µg)	**-**	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**	**-**	**-**	**-**
Volume of H_2O (ml)	**1.0**	**0.8**	**0.6**	**0.4**	**0.2**	-	**0.2**	**-**	**-**

Volume of buffered substrate (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**1.0**	**1.0**	**1.0**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**0.2**	**0.2**
Incubate at 37ºC for 15minutes									
Volume of NAD (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**0.2**
Volume of DNPH (ml)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Incubate at 37ºC for 15 minutes									
Volume of sodium hydroxide (ml)	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**
Optical density at 440 nm									

b. ASSAY THE ACTIVITY OF SERUM ACID PHOSPHATASE

AIM:

To assay the activity of serum acid phosphatase by method of king.

PRINCIPLE:

Serum acid phosphatase act on disodium phenyl phosphate at pH 4.9 liberating disodium phenol and phosphate. The liberated disodium phenol reacts with folin ciocalteau reagent and Na_2Co_3 to form a blue blue colored complex whose intensity can be measured at 650nm.

REAGENTS:

1. Stock solution

250mg of phenol is dissolved and made up to 250ml with 0.1N Hcl and stored in dark. Concentration = 1.0mg/ml

2. Working standard

To 1ml of stock solution, 20ml of folin ciocalteau reagent is added and made up to 100ml with distilled water kept in refrigerator. Concentration = µg/ml.

3. Folin ciocalteau reagent

100mg of sodium tungstate and 25mg of sodium molybdate are dissolved in 700ml of distilled water. 50ml of 85% per chloric acid and 100ml of con.Hcl are added and refluxed for 10hrs and cooled. 150g of lithium sulphate and 50ml of H_2O with a few drops of bromine water are added and boiled for 15minutes, cooled and made up to 1 litre and filtered. This solution should not have green tint. The solution should be kept in refrigerator.

4. Folin ciocalteau reagent

This reagent commercially available. It is diluted with distilled water 1:2 ratio.

5. Substrate

280mg of disodium phenyl phosphate is dissolved in 100ml of distilled water. Quickly boiled and cooled. Few drops of chloroform is added as preservative and kept in refrigerator.

6. Citrate buffer

21g of citric acid is dissolved in 100ml of NaoH and made up to 500ml with distilled water.

7. Buffered substrate

Equal volume of buffer and substrate are added. Prepare just before use.

8. 15% sodium carbonate

PROCEDURE:

0.2 to 1ml of phenol working standard of concentration ranging from 2 - 10µg are pipetted out into different test tubes and made up to 4ml with distilled water. Blank consist of 4ml of water and 2ml of sodium carbonate to the control (C) & test (T), 4ml of buffered substrate is added. 0.2ml of serum was added to test alone and incubated at 37°C for 1hr. Then 0.8ml of folin ciocalteau reagent is added to control and test. 0.2 ml of serum was added to the control and centrifuge and transferred 4ml of supernatant into another set of test tubes. 2ml of 50% Na_2Co_3 is added all test tubes. The intensity of the colour developed is read at 650nm after 15minutes. From the standard graph obtained enzyme activity is calculated.

Normal value

In adult male - 0.5 – 11.0 IU/L

In adult female – 0.2 – 9.5 IU/L

RESULT:

The serum acid phosphatase activity is ----------

Note

To convert kings unit into IU/L multiply by 1000 & divide by 94 (molecular weight of phenol)

To convert µ/L multiply by 10

To change minute to minute divide by 60

$$IU/L = \frac{KA \text{ units} \times 1000 \times 10}{94 \times 60} = KA \times 1.77$$

CALCULATION

Colorimeter reading ------ corresponds to ------ µg of phenol

4ml of supernatant liberated = ------ µg of phenol

6ml of supernatant liberated = ------ µg of phenol

= ------ µg of phenol

0.02ml serum liberated = ------ µg of phenol

To convert KA units into IU/L the following conversion is done.

REAGENTS	**B**	**S1**	**S2**	**S3**	**S4**	**S5**	**C**	**T1**	**T2**
Volume of working standard (ml)	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**-**	**-**	**-**
Concentration of working standard (µg)	**-**	**2.0**	**4.0**	**6.0**	**8.0**	**10.0**	**-**	**-**	**-**
Volume of H_2O (ml)	**4.0**	**3.8**	**3.6**	**3.4**	**3.**	**3.0**	**-**	**-**	**-**
Volume of buffered substrate (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**4.0**	**4.0**	**4.0**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**0.02**	**0.02**	**0.02**
Incubate at 37ºC for 1hour									
Volume of folin ciacalteau reagent (ml)	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**
Volume of serum (ml)							**0.2**		
Centrifuge									
Volume of supernatant (ml)							**4.0**	**4.0**	**4.0**
Volume of Na_2Co_3(ml)	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**
Optical density at 650 nm									

Test – control

=

c. ASSAY OF ACTIVITY OF SERUM ALKALINE PHOSPHATASE

AIM:

To assay the activity of serum acid phosphatase by method of moog (1946) and modified by king (1965).

PRINCIPLE:

Serum alkaline phosphatase catalyses the cleavage of phosphate at pH 10 it reacts with a the substrates disodium phenol and phosphate which gives a blue color complex with folin ciocalteau reagent and Na_2Co_3 due to presence of phenolic group. The intensity of blue colored developed is then read at 650nm.

REAGENTS:

1. Stock solution

250mg of phenol is dissolved and made up to 250ml with 0.1N Hcl and stored in dark. Concentration = 1.0mg/ml

2. Working standard

To 1ml of stock solution, 20ml of folin ciocalteau reagent is added and made up to 100ml with distilled water kept in refrigerator. Concentration = µg/ml.

3. Folin ciocalteau reagent

100mg of sodium tungstate and 25mg of sodium molybdate are dissolved in 700ml of distilled water. 50ml of 85% per chloric acid and 100ml of con.Hcl are added and refluxed for 10hrs and cooled. 150g of lithium sulphate and 50ml of H_2O with a few drops of bromine water are added and boiled for 15minutes, cooled and made up to 1 litre and filtered. This solution should not have green tint. The solution should be kept in refrigerator.

4. Folin ciocalteau reagent

This reagent commercially available. It is diluted with distilled water 1:2 ratio.

5. Substrate

280mg of disodium phenyl phosphate is dissolved in 100ml of distilled water. Quickly boiled and cooled. Few drops of chloroform is added as preservative and kept in refrigerator.

6. Carbonate buffer

6.36g sodium carbonate and 3.36g sodium bicarbonate are dissolved in 1L of distilled water.

7. Buffered substrate

Equal volume of buffer and substrate are added. Prepare just before use. The pH adjusted to 10.

8. 15% sodium carbonate

PROCEDURE:

0.2 to 1ml of phenol working standard of concentration ranging from 2 - 10µg are pipetted out into different test tubes and made up to 4ml with distilled water. Blank consist of 4ml of water and 2ml of sodium carbonate to the control (C) & test (T), 4ml of buffered substrate is added. 0.2ml of serum was added to test alone and incubated at 37°C for 1hr. Then 0.8ml of folin ciocalteau reagent is added to control and test. 0.2 ml of serum was added to the control and centrifuge and transferred 4ml of supernatant into another set of test tubes. 2ml of 50% Na_2Co_3 is added all test tubes. The intensity of the colour developed is read at 650nm after 15minutes. From the standard graph obtained enzyme activity is calculated.

Normal value – 25 – 100 IU/L

RESULT:

The serum acid phosphatase activity is ----------

Note

To convert kings unit into IU/L multiply by 1000 & divide by 94 (molecular weight of phenol)

To convert µ/L multiply by 10

To change minute to minute divide by 60

$$IU/L = \frac{KA\ units \times 1000 \times 10}{94 \times 60} = KA \times 1.77$$

CALCULATION

Colorimeter reading ------ corresponds to ------ µg of phenol

4ml of supernatant liberated = ------ µg of phenol

6ml of supernatant liberated = ------ µg of phenol

= ------ µg of phenol

0.02ml serum liberated = ------ µg of phenol

To convert KA units into IU/L the following conversion is done.

REAGENTS	**B**	**S1**	**S2**	**S3**	**S4**	**S5**	**C**	**T1**	**T2**
Volume of working standard (ml)	**-**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**-**	**-**	**-**
Concentration of working standard (µg)	**-**	**2.0**	**4.0**	**6.0**	**8.0**	**10.0**	**-**	**-**	**-**
Volume of H_2O (ml)	**4.0**	**3.8**	**3.6**	**3.4**	**3.**	**3.0**	**-**	**-**	**-**
Volume of buffered substrate (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**4.0**	**4.0**	**4.0**
Volume of serum (ml)	**-**	**-**	**-**	**-**	**-**	**-**	**0.02**	**0.02**	**0.02**
Incubate at 37ºC for 1hour									
Volume of folin ciacalteau reagent (ml)	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**
Volume of serum (ml)							**0.2**		
Centrifuge									
Volume of supernatant (ml)							**4.0**	**4.0**	**4.0**
Volume of Na_2Co_3(ml)	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**	**2.0**
Optical density at 650 nm									

d. ASSAY OF ACTIVITY OF SGOT (OR) ASPARTATE TRANSAMINASE

AIM

To determine the activity of aspartate transaminase in the given serum sample.

Principle

Aspartate transaminase catalyses the reverse interconversion between aspartate and glutamate and their 2 oxo analogues.

L-Aspartate +α-oxoglutarate $\xleftrightarrow{SGOT}$ Oxaloacetate + L-glutamate

The oxalo acetate is measured by colorimeter by a reaction with 2, 4 – dinitro phenyl hyrdrazine giving a brown coloured hydrazone derivative after the addition of 0.4N sodium hydroxide. The colour developed is read at 520 nm.

Reagents

1. Aspartic acid
2. α-keto glutaric acid
3. Pyruvic acid (sodium salt)
4. Sodium hydroxide
5. Disodium hydrogen phosphate
6. Dinitrophenyl hydrazine
7. Chloroform
8. Potassium hydrogen phosphate
9. Potassium dihydrogen phosphate
10. Phosphate buffer

Phosphate buffer (0.1M, pH 7.4)

Solution A

Disodium hydrogen phosphate – 14.2g/L

Solution B

Potassium dihydrogen phosphate – 13.6g/L

The buffer is prepared by mixing 420ml of Sol.A and 80 ml of sol.B.

SGOT Buffered substrate C:

Dissolve 2.66g of aspartic acid and 29.2mg of α keto glutaric acid in 1N NaOH and made up to 100 ml with phosphate buffer. The pH is adjusted to 7.4.

Dinitro phenyl hydrazine

20mg of dinitro phenyl hydrazine in 100 ml of 1N hydro chloric acid.

Stock pyruvate (20 mmol)

220mg of sodium pyruvate dissolved in 100 ml of phosphate buffer and stored in refrigerator. The concentration is 2.2mg.

Working standard

10 ml of stock standard is diluted to 100 ml with phosphate buffer. The concentration is 220µg/ml which is 20µm/ml.

PROCEDURE

Into a series of test tubes pipette out 0.1 to 0.5ml of working standard pyruvate solution corresponding to the concentration range 02 to 1.0 µg/ml and made up the volume in all the test tubes to 1.0 ml by adding buffered substrate. 1ml of buffered substrate is taken in different test tubes labelled as control and test. 0.2 ml of serum is added to the test alone.

0.2ml of phosphate buffer is added to all the test tube and the std tubes are incubated at 37^0 C for 30 minutes and the control and test are incubated for 1hrs.

At the end of the incubation 1ml of DNPH is added to all the test tubes and 0.2 ml of serum is added to control, tubes were incubated at 37^0 C for 15mins.

Then 10ml of sodium hydroxide is added to all the tubes and the brown colour development is readed at 540nm.

From the graph obtained by plotting concentration range on x axis and the corresponding optical density values on y axis. The enzyme activity is calculated.

Normal range – 3-16IU/Litre.

RESULT

The serum aspartate transaminase activity was found to be ----------

CALCULATION

Colorimeter reading ___ corresponds to ____ µm of pyruvate

That is 0.2 ml of serum contains ___ µm of pyruvate

Therefore 1000 ml of serum liberate $\frac{0.1 \times 1000}{0.2}$

= ____ µm of pyruvate

Activity of SGOT is given by $\frac{=}{60}$

= _____ IU/litre

REAGENTS	B	S1	S2	S3	S4	S5	C	T1	T2
Volume of working standard (ml)	-	0.1	0.2	0.3	0.4	0.5	-	-	-
Concentration of working standard (µg)	-	0.2	0.4	0.6	0.8	1.0	-	-	-
Volume of buffered substrate (ml)	1.0	0.9	0.8	0.7	0.6	0.5	1.9	1.0	1.0
Volume of phosphate buffer (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Volume of serum (ml)	-	-	-	-	-	-	-	0.2	0.2
Incubate at 37ºC for one hour									
Volume of DNPH (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of serum (ml)	-	-	-	-	-	-	0.2	-	-
Incubate at 37ºC for 15 minutes									
Volume of sodium hydroxide (ml)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Optical density at 540 nm									

e. ASSAY THE ACTIVITY OF SGPT (OR) ALANINE TRANSAMINASE

AIM:

To assay the activity of alanine amino trasferase in serum.

PRINCIPLE:

When serum is incubated with substance containing α keto glutarate and L-alanine L-glutamate and pyruvate are formed.

The enzyme catalyses the following reaction:

L-Alanine + α-oxoglutarate ⟶ Pyruvate + L-glutamate

Pyruvate so formed is coupled with 2, 4- dinitro phenylhydrazine to give the corresponding hydrazone which gives a brown colour which is measured at 540 nm.

REAGENTS

- L alanine
- α-keto glutaric acid
- Pyruvic acid (sodium salt)
- Sodium hydroxide
- Disodium hydrogen phosphate
- Potassium dihydrogen phosphate
- Di nitro phenyl hydrogen

1. Phosphate buffer (0.1M, pH 7.4)

   Add 11.3g dry anhydrous disodium hydrogen phosphate and 2.7g dry anhydrous potassium dihydrogen phosphate in one litre volumetric flask and make up to the mark with water. Check the pH and store at 4ºC.

2. Buffered substrate:

   Dissolve 1.78g of alanine and 29.2mg of α keto glutaric acid in 1N NaOH and made up to 100 ml with phosphate buffer. The pH is adjusted to 7.4.

3. Dinitro phenyl hydrazine

   20mg of dinitro phenyl hydrazine in 100 ml of 1N hydro chloric acid.

4. Stock pyruvate (20 mmol)

   220mg of sodium pyruvate dissolved in 100 ml of phosphate buffer and stored in refrigerator. The concentration is 2.2mg.

5. Working standard

   10 ml of stock standard is diluted to 100 ml with phosphate buffer. The concentration is

220µg/ml which is 20µm/ml.

6. 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water

PROCEDURE:

0.1 – 0.5 ml of pyruvate standard of concentration range 0.2 – 1µm is pipette out in to a series of test tubes. The volume is made up to 1.0 ml with buffered substrate. 1.0 ml of buffered substrate serves as blank.

Added 1.0 ml of buffered substrate and 0.2 ml of phosphate buffer to the test and control tubes. Added0.2 ml of serum to the test alone. The standards, test and control were incubated along with the blank at 37^{o}C for 30 minutes. At the end of incubation period added 1.0 ml of 2, 4 dinitro phenyl hydrazine to all the tubes. To the control tube added 0.2 ml of serum after arresting the reaction with 2, 4 dinitro phenyl hydrazine. All the tubes are incubated at 37^{o}C for 15 minutes. At the end of incubation period added 10.0 ml of 0.4N NaOH to all the tubes. The brown colour developed was read at 540 nm after 10 minutes.

From the standard graph obtained by plotting the concentration of pyruvate on x axis and optical density value on y axis the enzyme activity is measured.

Normal value: 4 – 17IU/litre.

RESULT:

The activity of SGPT in serum is found to be _____ IU/litre.

REAGENTS	B	S1	S2	S3	S4	S5	C	T1	T2
Volume of working standard (ml)	-	0.1	0.2	0.3	0.4	0.5	-	-	-
Concentration of working standard (µg)	-	0.2	0.4	0.6	0.8	1.0	-	-	-
Volume of buffered substrate (ml)	1.0	0.9	0.8	0.7	0.6	0.5	1.9	1.0	1.0
Volume of phosphate buffer (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Volume of serum (ml)	-	-	-	-	-	-	-	0.2	0.2
Incubate at 37ºC for about 30 minutes									
Volume of DNPH (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of serum (ml)	-	-	-	-	-	-	0.2	-	-

Incubate at 37°C for 15 minutes									
Volume of sodium hydroxide (ml)	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**
Optical density at 540 nm									

CALCULATION

Colorimeter reading ___ corresponds to ____ µm of pyruvate

That is 0.2 ml of serum contains ___ µm of pyruvate

Therefore 1000 ml of serum liberate $\frac{0.1 \times 1000}{0.2}$

= ____ µm of pyruvate

Activity of SGOT is given by $\frac{\quad}{30}$

= _____ IU/litre

f. ASSAY OF ACTIVITY OF 5'– NUCLEOTIDASE

AIM

To assay the activity of 5' – nucleotidase in the given serum sample.

PRINCIPLE:

The enzyme hydrolyses nucleotide with the phosphate group on 5' c – carbon atom of the ribose sugar.

Adenosine – 5 – phosphate ⟶ adenosine + phosphate

This nucleotide was hydrolyzed by non-specific phosphatase such as alkaline phosphatase in serum. However 5'-nucleotidase activity is inactivated by Ni so that the hydrolysis is carried with or without Ni, the difference gives 5'-nucleotidase activity.

REAGENTS:

1. Tris buffer (20mm, pH- 7.5)

2.422g of tris is dissolved in 680ml of distilled water. The pH is adjusted to 7.5 with Hcl and NaoH and made up to 1 liter with water.

2. Adenosine 5'- phosphatase solution

Addede 9ml of NaoH (100mm) to 174mg adenosine phosphate and made up to 50ml and stored in refrigerator.

3. $MnSO_4$

20mM/litre, 4.66g/L

4. Trichloro acetic acid (100g/L) 10%

5. Acetate buffer (2µm/L) pH-4)

46g of anhydrous solution of sodium acetate and 2.5g of $CuSO_4$ was dissolved in 1litre of acetic acid.

To prepare 2M/L, acetic acid, glacial acetic acid 115ml was made up to 1litre and standardized against NaoH using phenolphthalein indicator.

6. Ammonium molybdate solution (50%)

50g/L of water

7. Metol sulphite solution

2mg of metol and 10g sodium sulphite dissolved in water and made up to 100ml. kept in dark at 4°C.

8. Phosphate stock solution

Stock solution containing 6Mm//L was prepared by dissolving 206mg of anhydrous potassium dihydrogen phosphate in water and made up to 250ml, yellowed by a drop of chloroform.

9. Working standard

1ml of stock was made up to 100ml with distilled water. Concentration = 0.06M/L.

10. Nickel chloride solution

23.77g of nickel chloride in distilled water was made up to 1L with the same.

PROCEDURE:

Total activity

Taken two test tubes labelled 'C' and 'T'. Added 0.1ml of serum to 'T' tube alone. Then added 0.1ml of manganese sulphate 1.5ml of tris buffer to both the tube and incubated at 37°C.

At the end of incubation o.2ml of adenosine monophosphate solution and allowed to stand. Then add 2ml of TCA,mixed well and allowed to stand for 5minutes.

Transfer 2ml of supernatant into a new set of test tube. Add 3.0ml of acetate buffer, 0.5ml of ammonium molybdate and 0.5ml of metol sulphate to the test tubes. Mix well and read the colour developed at 680nm.

Non – specific alkaline phosphatase activity

Two test tubes labelled control and test were taken and treated in the same way as for the assay of total activity. The only difference between the two procedure is that Nickel chloride is added. In this procedure prior to first incubation. Unlike the addition after incubation as in the case of the previous procedure.

Simultaneously a set of standard run by pipetting out 0.04 – 2ml of working standard to the concentration range 0.02 to 0.12 Mm in to a series of test tubes. The volume in all the tubes were made up to 2ml with distilled water. Then 3.0 ml of acetate buffer 0.5 ml of ammonium molybdate and 0.5ml of metal sulphate are added to all the tubes. A blank is maintained alone with 2ml of water. The blue color developed in all the tubes were read at 680nm after 20minutes.

A standard graph is drawn by plotting the concentration of phosphate on X – axis and optical density on Y-axis. From the graph the activity of 5'nucleotidase can be calculated.

Normal value – 2 – 17 IU/L

RESULT:

The activity of 5'- nucleotidase per minute was found to be ----------

CALCULATION:

Total activity non - specific of alkaline phosphatase-------
Optical density reading 0.02 corresponds to---------- of phosphate.
2.0 ml of serum supernatant liberated---------- µm of phosphate.
4.0 ml of serum supernatant corresponds to 0.1ml of serum.
Therefore 0.01ml serum liberated -------- µm of phosphate.
1000ml of serum liberate----------
Activity of 5'- nucleotidase per minute----------

REAGENTS	B	S1	S2	S3	S4	S5
Volume of working standard (ml)	-	0.4	0.8	1.2	1.6	2.0
Concentration of working standard (µg)	-	0.024	0.048	0.072	0.098	0.122
Volume of water (ml)	2.0	1.6	1.2	0.8	0.4	-

Volume of acetate buffer (ml)	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**
Volume of ammonium molybdate (ml)	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**
Volume of metal sulphate (ml)	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**
Blue color developed was read at 680nm						
Optical density at 680 nm						

g. SODIUM POTASSIUM ATPASE

Principle

Na^+/K^+ ATPase transports Na^+ and K^+ against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). Na^+ K^+ ATPase activity was estimated from the amount of Pi liberated. The inorganic phosphorous liberated is estimated by Fiske and Subbarow method.

Reagents

- 184 mM Tris-HCl buffer, pH 7.5
- 50 mM $MgSO_4$
- 50 mM KCl
- 60 mM NaCl
- 1 mM EDTA
- 40 mM ATP

Procedure

1.0 ml of Tris buffer and 0.2 ml of each of the above reagents were mixed, together. Thus the assay medium in a final volume of 2.0 ml, contained 92 mM tris buffer, 50 mM $MgSO_4$, 60 mM NaCl, 1 mM EDTA and 4 mM ATP. After 10 minutes, equilibrium at 37˚C in an incubator, reaction was started by the addition of 0.1 ml of homogenate. The assay medium was incubated for 15 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The phosphorus content in the supernatant was estimated by the method of Fiske and Subbarow. The enzyme activity is expressed as micromoles of Pi liberated/min/mg protein.

f. DETERMINATION OF CERULOPLASMIN

AIM:

To determine the activity of ceruloplasmin by estimating by estimating its activity as oxidase or paraphenyl diamine

PRINCIPLE:

Amine oxidase activity of ceruloplasmin is measured by paraphenyl diamine as substrate. Non – enzymatic oxidation is prevented by sodium azide. Thus the assay is carried out and the oxide measures non – enzymatic oxidation. The difference is the measure of the rate of enzyme catalyzed by oxidation of diamine.

REAGENTS:

1. Para phenyl diamine – 0.5% in distilled water.
2. Acetate buffer – 0.4M, pH -5.5 – 12.0ml of 1M acetic acid is added to 200ml of 1M sodium acetate
3. Sodium azide

0.5% in distilled water.

PROCEDURE:

0.1ml of serum preferby fresh, free from hemolysis is taken in three test tubes, one 'control' and two 'test'. 1.0ml of sodium Azide was added. To control, then added 8.0ml of acetate buffer to all the tubes followed by 1.0ml of paraphenyl diamine to both 'control' and 'test' tubes. Mixed well and placed in an incubator for one hour at 37°C. after incubation 1.0ml of sodium azide is added to the test and mixed well.

Cool the tubes at 4°C to 10°C for 10minutes. Read the color developed at 530nm.

RESULT:

The ceruloplasmin activity was found to be ----------

Volume of serum (ml)	**Volume of sodium azide (ml)**	**Volume of acetate buffer(m)**	**Volume of paraphenyl diamine (ml)**		**Volume of sodium azide (ml)**		**Optical density 530nm**
Test				Kept in		Kept 4°C -	
0.1	-			incubation	1.0	10°C for	
0.1	-	8.0	1.0	at 37°C	1.0	30minutes	
Control				for 1hr			
0.1	1.0						

Ceruloplasmin activity = optical density of (test – control)

IMMUNOLOGY (DEMONSTRATION)

Experiment. 2

RAISING OF ANTIBODIES- SINGLE SOLUBLE AND PARTICULATE ANTIGEN

This protocol describes isolation of live peripheral blood lymphocytes (PBL) for

subsequent use in translational research. The procedure is compatible with modern molecular techniques (e.g. RNA & DNA, and protein isolation for genomics & proteomics) and use of live cells for subsequent in vitro experiments.

Method:

- 10 ml blood with EDTA as anticoagulant and 10 ml blood with heparin
- Separate lymphocytes and monocytes from other blood components using the
- Ficoll method:
- Dilute the blood with sterile PBS 1:1.
- Add 10 ml of Ficoll in a centrifuge tube, (the proportion between Ficoll and blood should be 1/3 and 2/3, respectively)

- Carefully pour the diluted blood onto the ficoll solution (the blood must remain on top, do not mix).

- Centrifuge the tubes 20 min at 1600rpm (350g).
- Harvest the ring with white blood cells without touching the Ficoll using a sterile pipette tips.

- Dilute the white blood cells with PBS, then wash them twice in PBS.
- Resuspend the pellet in 1.5 ml FCS containing 10% DMSO. Put in 2 cryotubes. (For purposes not requiring live lymphocytes (e.g. DNA & RNA

  isolation) the pellet can be frozen directly)

- Mark Heparin or EDTA pretreatment on the label.
- Store PBL at -80°C.

Purification of Immunogloulin

Antibody purification with Protein A

To accomplish antibody purification with Protein A, Protein G, Protein A/G or Protein L, they are covalently immobilized onto porous resins (such as beaded agarose) or magnetic beads. Because these proteins contain several antibody-binding domains, nearly every individual

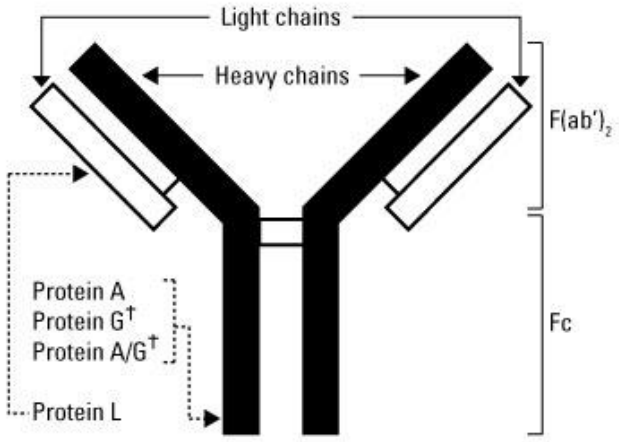
immobilized molecule, no matter its orientation maintains at least one functional and unhindered binding domain. Furthermore, because the proteins bind to antibodies at sites other than the antigen-binding domain, the immobilized forms of these proteins can be used in purification schemes, such as immunoprecipitation, in which antibody binding protein is used to purify an antigen from a sample by binding an antibody while it is bound to its antigen.

Protein A, G, A/G and L have different binding properties, which make each one suitable for different types of antibody targets (e.g., antibody subclass or animal species). It is important to realize that use of Protein A, G or L results in purification of general immunoglobulin from a crude sample. Depending on the sample source, antigen-specific antibody may account for only a small portion of the total immunoglobulin in the sample. For example, generally only 2–5% of total IgG in mouse serum is specific for the antigen used to immunize the animal.

Using a column of Protein A agarose resin and rabbit serum as the example, the general procedure for antibody purification with these ligands is as follows:

Bind: Add a clarified, physiologic-buffered (pH 7 to 8) sample of rabbit serum to the column and allow it to slowly pass through or mix with the Protein A resin to allow the IgG to bind to the immobilized ligand.

	Protein A
Species	*Staphylococcus aureus*
Human Pathology	Component of human body flora; cause of "Staph" infections
Native Size(s)	40 to 60kDa (variable numbers of repeated domains)
Binding Domains	5 for IgG (most common form)
Ig-binding Target	heavy chain constant region (Fc) of IgG (CH2-CH3 region)



Binding sites of antibody-binding proteins. Proteins used to immobilize antibodies to beaded support show specificity to different antibody domains. Protein A and G bind to the heavy chains of the antibody Fc region, while Protein L specifically binds the light chains of the two Fab regions of the F(ab')2 antibody fragment. † Protein G can also bind Fab fragments in certain conditions.

Wash: Add phosphate-buffered saline (PBS) and allow it to pass through the column to wash away nonbound serum components. Use a volume of wash buffer equivalent to 5 to 10 times the resin volume.

Elute: Add acidic elution buffer (e.g., 0.1M glycine-HCl, pH 2.8), and collect small fractions of solution that pass from the column. The low-pH condition dissociates the antibody from the immobilized Protein A, and the IgG is recovered in its purified state in one or several of the collected fractions.

Neutralize or exchange buffer: Use a protein assay or other means to identify and combine elution fractions that contain the purified antibody. Then add 1/10th volume of 1M Tris-HCl (pH 8.5) to neutralize the buffer. Alternatively, use a desalting column or dialysis to exchange the purified antibody into a more suitable buffer for long-term storage.

Experiment .3

IMMUNODIFFUSION- SINGLE RADIAL AND DOUBLE DIFFUSION

AIM:

To study the immunodiffusion technique by Single Radial Immunodiffusion

INTRODUCTION:

Single Radial Immunodiffusion, also known as Mancini technique, is a quantitative immunodiffusion technique used to detect the concentration of antigen by measuring the diameter of the precipitin ring formed by the interaction of the antigen and the antibody at optimal concentration. In this method the antibody is incorporated into the agarose gel whereas the antigen diffuses into it in a radial pattern.

PRINCIPLE:

Single Radial Immunodiffusion is used extensively for the quantitative estimation of antigen. Here the antigen-antibody reaction is made more sensitive by the addition of antiserum into the agarose gel and loading the antigen sample in the well. As the antigen diffuses into the agarose radially in all directions, it's concentration continuously falls until the equivalence point is reached at which the antigen concentration is in equal proportion to that of the antibody present in the agarose gel. At this point ring of precipitation ('precipitin ring') is formed around the well. The diameter of the precipitin ring is proportional to the

concentration of antigen. With increasing concentration of antigen, precipitin rings with larger diameter are formed. The size of the precipitin rings depend on Antigen concentration in the sample well Antibody concentration in the agarose gel Size of the sample well Volume of the sample

Radial Immunodiffusion (Mancini)

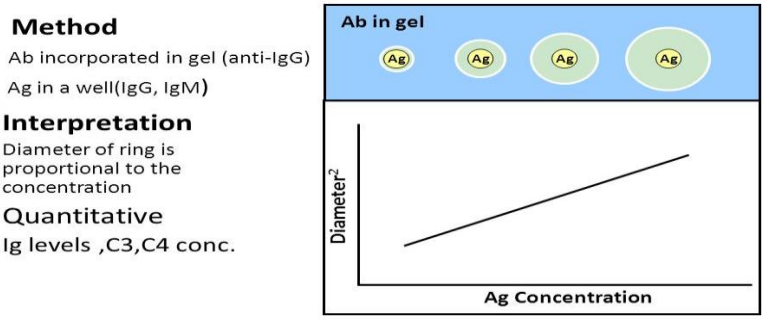


Fig 1: In Single Radial Immunodiffusion assay the diameter of the precipitin ring increases with increasing concentration of the antigen Thus, by having various concentrations of a standard antigen, standard curve can be obtained from which one can determine the amount of an antigen in an unknown sample. Thus, this is a quantitative test. If more than one ring appears in the test, more than one antigen/antibody reaction may have occurred. This could be due to a mixture of antigens or antibodies.

1.Prepare 10 ml of 1% agarose gel (as give in the important instructions). Take 6 ml of this gel solution in a clean test tube.

2. Allow the solution to cool down to 55-60oC and add 80 µl of antiserum to 6 ml of agarose solution. Mix well for uniform distribution of the antibody.

3.Pour agarose solution containing the antiserum on to a grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

4.Place the glass plate on the template provided.

5.Punch wells with the help of gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming rugged wells.

6.Add 10 µl of the given standard antigen and test antigen samples to the wells.

7. Incubate the glass plate in a moist chamber overnight at 37oC.

OBSERVATION AND RESULT:

Observe for precipitin rings surrounding the antigen wells (Fig 3). Mark the edges of the precipitin rings and measure the diameter of the rings as shown in tabl

Experiment .4

IMMUNOELECTROPHORESIS

AIM:

To check antisera for the presence of antibody for a particular antigen and to characterisc the nature of the antibody by electrophoresis.

PRINCIPLE:

The technique is based on the principles of electrophoresis of antigens and immunodiffusion of the electrophoresis antigen with poly specific antiserum to form precipitin bands. When antigens are subjected to electerophoresis in an agarose gel, they are separated according to their acquired size, charge and shape by migrating the different positions. Antigens thus dissolved by electerophoresis are subjected to immune diffusion with anti serum added in a trough cut in the agarose gel. Due to diffusion, density gradient of antigen and antibody are formed and at the zone of equivalence antigen – antibody are formed and at the zone of equivalence antigen – antibody complex precipitate to form an opaque arch shaped line in the gel. The precipitin line indicates the presence antibody specific too the antigen.

MATERIALS REQUIRED

Conical flask, measuring cylinder, test tubes, glass plate, gel punch, with syringe, template, micropipette tips, moist chamber, agarose assay buffer, antigen, test antisera.

PROCEDURE:

Preparation of gel plates:

1. Prepare 10.0ml of 1.5% agarose (0.15gm/10.0ml) in 1x electrophoresis buffer by heating slowly till agarose dissolves completely.
2. Mark the end of the glass plate will be towards negative electrode during electrophoresis.
3. Pour 10.0ml of agarose solution to a clean glass slide placed on the horizontal surface and allow the gel to set.
4. Place the gel plate on the template provided. Punch well within the gel with the help of a gel punch corresponding to the markings on the template towards the negative end.
5. Cut troughs with the gel cutter provided.

IMMUNOELECTROPHORESIS

1. Add 12 - 15µl of antigen to the well.
2. Place the glass plate in electrophoresis stand such that the antigen well is at a negative electrode. Pour the electrophoresis buffer such that it covers the gel.
3. Set the voltage to 50 – 100 volts and electrophoresed until the blue dye travel 3-4 cm from the well. Do not elephoresce beyond 3hrs**.**

RESULT:

The presence of more than one precipitate line indicates the heterogenesity of antiserum antigen.

Experiment .5

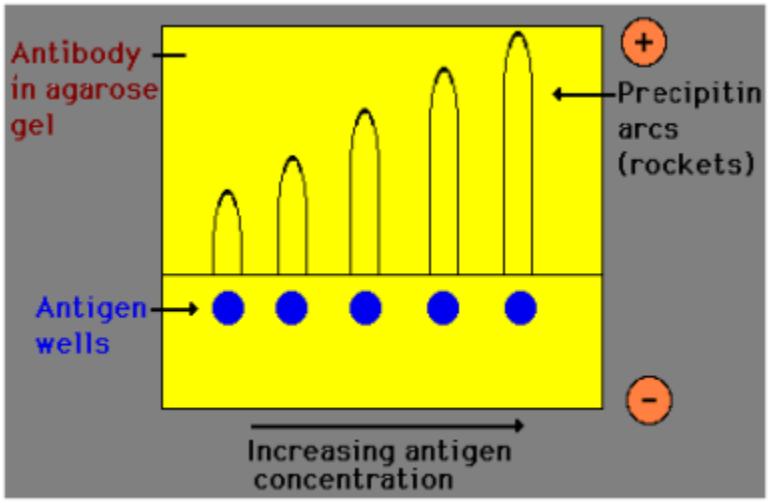
ROCKET ELECTROPHORESIS

AIM:

To determine the concentration of antigen using a novel method of lauret (1965).

PRINCIPLE:

In Rocket Immunoelectrophoresis, negatively charged antigen samples are electrophoresed in an agarose gel containing antibody which is specific to that antigen. As the antigen moves out of the well and enters the agarose gel, it combines with the antibody to form immune complex which is visible as white. Because the antigen is migrated through the gel under the influence of an applied electric current, it moves in one direction. During the initial phase there is considerable antigen excess over antibody and no visible precipitation occurs. However, as the antigen sample migrates further through the agarose gel, more antibody molecules are encountered that interact with the antigen to form immune complex. When this immune complexes become large enough to be retained within the gel, movement of the antigen stops. The area of precipitin has the shape of a rocket and its height is proportional to the concentration of antigen in the corresponding well.



MATERIALS REQUIRED:

Glass wares: Conical flask, Measuring cylinder, Beaker Reagents: Sterile distilled water, alcohol Other requirements: Incubator (37o C), Microwave or Bunsen burner, Vortex mixer, spatula, Micropipettes, Tips, Moist chamber (box with wet cotton)

PROCEDURE:

1. Prepare 15 ml of 1 % agarose (as given in important instructions).

2. Cool the solution to 55-60o C and add 250 µl of antiserum to 13 ml of agarose solution. Mix well for uniform distribution of antibody.

3. Pour agarose solution containing the antiserum on to a grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.

4. Place the glass plate on the template provided.

5. Punch wells with the help of gel puncher. Use gentle suction to avoid forming rugged wells.

6. Add 10 µl of the given standard antigen and test antigen samples to the wells.

A. Standard Antigen A (1.87 mg/ml)

B. Standard Antigen B (0.94 mg/ml)

C. Standard Antigen C (0.47 mg/ml)

D. Standard Antigen D (0.23 mg/ml)

E. Test Antigen 1

F. Test Antigen 2

7. Pour 1X TBE buffer into the electrophoresis tank such that it just covers the gel.

8. Electrophorese at 80-120 volts and 60-70 mA, until the blue dye travels 3-4 cms from the well. Do not electrophorese beyond 3 hours, as it is likely to generate heat.

9. Incubate the glass plate in a moist chamber overnight at 37o C.

RESULT:

Observe for precipitin peaks in the shape of 'Rocket' formed against a dark background. Mark the tip of the precipitin peaks and measure the peak height from the upper edge of the well to the tip of the peak.

Experiment.6

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

DESCRIPTION:

An enzyme-linked immunosorbent assay (ELISA) is used to detect the presence of an antigen in a sample. The antigen is immobilized to the well of a plate by adsorption, or captured with a bound, antigen-specific antibody. A detection antibody is then added forming a complex with the antigen, if present. The detection antibody can be covalently linked to an enzyme, or itself be detected by a secondary, enzyme linked antibody. Enzyme substrate is then added to the wells producing a visible signal that is correlated with the amount of antigen and measured by a spectrophotometer.

PROCEDURE:

- 100µl peptide (@4µg/ml) in coating buffer is added to individual wells of a microtiter plate. Incubate the plate for 2 hours at 37°C or overnight at 4°C.
- Remove the coating solution and wash the plate three times by filling the wells with 100 µl PBS-0.05%Tween20. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.
- Block the remaining protein-binding sites in the coated wells by adding 100µl blocking buffer, 3% skim milk in PBS per well. Incubate for 1 hour at RT with gentle shaking.
- Wash the plate three times with 100ul PBS-0.05% Tween 20.
- Add 50µl of diluted antibody to each well. Incubate the plate at 37℃ for an hour with gentle shaking.
- Wash the plate six times with 100ul PBS-0.05%Tween 20.
- Add 50µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use. Incubate at 37℃ for an hour.
- Wash the plate six times with 100ul PBS-0.05%Tween20.

- Prepare the substrate solution by mixing acetic acid, TMB and 0.03% H2O2 with the volume ratio of 4:1:5.
- Dispense 50µl of the substrate solution per well with a multichannel pipe. Incubate the plate at 37℃ in dark for 15-30mins.
- After sufficient color development, add 100µl of stop solution to the wells (if necessary).
- Read the absorbance (optical density at 450nm) of each well with a plate reader.