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# Detection of Protein by Microdrop Analysis

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**Detection of Protein by Microdrop Analysis**

A Project

Submitted

To

Governors State University

By

Yogeshkumar Radadiya

In

Partial Fulfillment of the

Requirements for the Degree

Of

Masters in Science in Analytical Chemistry

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Governors State University

University Park, Illinois

Dedicated to my parents

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**Abstract :**

Analysis of protein depends particularly on protein concentration<sup>1</sup>. Protein concentration measurement is the most important part in the research work to conduct protein-related studies. Although there are many methods available for this purpose, each method has certain limitations. The aim of the experiment is to develop either new or modified analytical method for the analysis and detection of protein using newly introduced micro-plate reader equipped with Take-3 microplate with the help of Gen-5 software. An ideal assay should be simple and easy to carry out. Another aspect to be taken into consideration are low inference, stability of measured components and low protein to protein variation<sup>2</sup>. This research work involves to measure protein concentration using various assays such as, absorbance at 280 nm, Bradford assay and BCA assay. Bradford and BCA assays are the most popular tools to quantify the protein sample. Bradford assay involves the measurement of absorbance at 595 nm<sup>3</sup>. BCA assay involves measurement of absorbance at 562 nm. BSA was used as protein standard because it is highly pure and inexpensive. The overall strategy is to develop a robust assay that uses the least amount of sample and test reagents . Successful completion of this work will aid protein researchers in quick identification and analysis of proteins.

### **Introduction :**

Because of wide range of protein functions, it is necessary to determine the concentration of protein in complex mixture. Estimation of concentration is necessary for protein analysis, protein purification, to study cells and other research applications<sup>4</sup>. Protein assays are the most convenient tools for this purpose. There are many methods available to determine protein content, such as biuret assay, weight analysis, absorbance assays; each method is having certain limitation<sup>5</sup>. Below are the main two types of protein assays.

1. Absorbance assays i.e absorbance at 205 nm, absorbance at 280 nm, extinction coefficient
2. Colorimetric assays i.e biuret assay, BCA assay, Bradford assay, modified lowry assay.

All assays involve estimation of protein concentration from measurements obtained from serial dilutions of a standard solution. The selection of assay depends on compatibility with the sample, sample volume, level of sensitivity ,presence or absence of interfering agents<sup>4</sup>. Among these various methods, colorimetric methods are commonly used because they offer more accuracy and convenience<sup>6</sup>.

This research work involves the use of various methods to quantitate the protein sample such as absorbance at 280 nm, Bradford assay and BCA assay. Bradford and BCA assays were performed at different incubation times to see any changes in the sensitivity of the assays.

Absorbance at 280 nm :

It is the simple and fast method for the purpose of protein quantitation as it does not require any additional reagents.

### Bradford Assay :

It is the dye binding assay that involves the change in the color of the dye which changes accordingly with the change in the concentration of a protein. Bradford assay involves the use of Coomassie Blue G-250 dye. It is the dimethylated form of Coomassie Brilliant Blue dye and it has a greenish tint.

Chemical properties of dye are as follows :

Chemical formula :  $C_{47}H_{48}N_3NaO_7S_2$

Molecular weight : 854.02 g/mol

Coomassie dye exists in three forms – cationic (red colored) , neutral ( green colored ) and anionic ( blue colored )<sup>7</sup>. Under acidic conditions, dye is in cationic form( $\lambda_{max} = 470$  nm). When it is bound to protein, it is converted to blue-colored complex which is measured at 595 nm<sup>3, 8-9</sup>.

### BCA assay :

Chemical properties of Bicinchoninic acid are as follows :

Chemical formula :  $(HO_2CC_9H_5N)_2$

Molecular weight : 344.33 g/mol

The assay involves mainly two reactions. The first reaction is a biuret reaction. The solution used is alkaline solution. Firstly peptide bonds in protein act on copper ions and reduce  $Cu^{+2}$  ions to  $Cu^+$  ions. The amount of reduction is proportional to the amount of protein present in the sample.



This step is temperature dependent. In the second step, two molecules of BCA form complex with  $\text{Cu}^+$  ion leading to the formation of a purple colored solution which is measured at 562 nm<sup>10-11</sup>.

### **Experimentation :**

#### **Instruments :**

1. Epoch Microplate spectrophotometer : It has 200 nm to 999 nm wavelength range with a microplate reading capacity of 6 to 384. It is also included with Gen5 microplate data analysis software. Another feature is that it offers compatibility with Take-3 microplate which can be used for low volume protein quantification.
2. Humidified incubator : It is used to incubate the protein mixture.
3. Micro-pipette

#### **Materials and reagents :**

1. Bovine Serum Albumin ( lot # 119K1673 ) from Sigma Aldrich.
2. BCA kit : Bicinchoninic acid solution , Copper(II)sulfate solution from Sigma Aldrich
3. Coomassie blue G-250 dye ( lot # KD132217 ) from Thermo Scientific
4. Phosphate-buffered saline ( lot # 21040174 ) from Mediatech, Inc.
5. Parafilm "M"

### **Preparation of Reagents :**

- Preparation of BSA standards :

To plot a standard curve of protein concentration vs absorbance, it is necessary to make a series of dilutions of BSA protein standards. BSA is preferred for the protein assay because it is highly pure and inexpensive. 20 mg of BSA is dissolved in 10 ml of PBS solution from which different concentrations of BSA (blank , 0.0625 mg/ml , 0.125 mg/ml , 0.25 mg/ml , 0.5 mg/ml , 1 mg/ml , 1.5 mg/ml , 1.75 mg/ml , 2 mg/ml ) are prepared.

- Preparation of Bradford reagent :

500 uL of Coomassie blue G-250 dye is pipetted into appender tube.

- Preparation of BCA reagent :

490 uL of Bicinchoninic acid solution is pipetted into appender tube through micro-pipette and 10 uL of Copper(II)sulfate solution is added to this solution.

### **General Methodology :**

#### **Absorbance at 280 nm :**

1. 5 uL of different BSA standards ( blank , 0.125 , 0.25 , 0.5 , 0.75 , 1 , 1.5 , 2 mg/ml ) are pipetted onto parafilm™M”.
2. Incubate the mixture for 5 min.
3. Measure the absorbance at 280 nm.

**Bradford Assay :**

1. 5 uL of different BSA protein standards are pipetted onto parafilm™ on which 5 uL of Coomassie blue G-250 is added. The mixture is mixed with the help of a micro-pipette. It is then kept at a room temperature for a period of 5 min.
2. Then 5 uL of the mixture is placed on Take-3 microplate.
3. Measure absorbance at 595 nm.

**BCA assay :**

1. 5 uL of different BSA standards are pipetted on a parafilm™ and 5 uL of BCA reagent is added on each protein standard. It is thoroughly mixed with the help of a micro-pipette. The mixture is then allowed to keep at the room temperature for 5 min.
2. 5 uL of the mixture is pipetted through on a Take 3 micro-plate.
3. Measure the absorbance at 562 nm

**Results :**

Table : 1 - Absorbance at 280 nm :

Conc ( mg/ml)	Absorbance at 280 nm
0	0
0.125	0.006
0.25	0.001
0.5	0.012
0.75	0.018
1	0.029
1.5	0.045
2	0.058

Table : 2 - Bradford assay at room temperature with 5 min of incubation time :

Conc ( mg/ml )	Abs at 595 nm
0	0
0.0625	0.04
0.125	0.045
0.25	0.05
1	0.06
1.5	0.063
2	0.065

Table : 3 - Bradford assay at room temperature with incubation times of 5 and 30 min.

Conc ( mg/ml )	Abs at 595 nm after 5 min	Abs at 595 nm after 30 min
0	0	0
0.0625	0.041	0.042
0.125	0.045	0.046
0.25	0.049	0.052
1	0.062	0.064
1.5	0.065	0.068
2	0.069	0.072

Table : 4 - BCA Assay with low concentration ( 5 min incubation time )

Conc ( mg/ml)	Abs at 562 nm
0	0
0.0625	0.002
0.125	0.016
0.25	0.03
0.5	0.043
1	0.082
1.5	0.115
2	0.131

Table : 5 - BCA Assay with low concentration ( 30 min of incubation time )

Conc ( mg/ml )	Abs at 562 nm ( 30 min )
0	0
0.0625	0.004
0.125	0.024
0.25	0.046
0.5	0.08
1	0.132
1.5	0.189
2	0.225



Table : 6 - BCA assay at room temperature at the interval of 5 min :

Conc (mg/ml)	Absorbance at 562 nm						
	( 0 min )	5 min	10 min	15 min	20 min	25 min	30 min
0	0	0	0	0	0	0	0
0.125	0.015	0.018	0.02	0.022	0.023	0.024	0.025
0.25	0.019	0.028	0.032	0.035	0.038	0.041	0.043
0.5	0.027	0.048	0.056	0.062	0.067	0.072	0.075
0.75	0.036	0.062	0.072	0.08	0.086	0.094	0.099
1	0.045	0.082	0.098	0.107	0.116	0.124	0.13
1.5	0.055	0.089	0.108	0.121	0.133	0.145	0.153
2	0.069	0.132	0.157	0.175	0.191	0.208	0.221

Table : 7 – BCA assay in humidified incubator at the interval of 5 min .

Conc (mg/ml)	Absorbance at 562 nm						
	0 min	5 min	10 min	15 min	20 min	25 min	30 min
0	0	0	0	0	0	0	0
0.125	0.022	0.03	0.032	0.033	0.035	0.034	0.036
0.25	0.033	0.047	0.05	0.054	0.056	0.056	0.06
0.5	0.043	0.071	0.07	0.072	0.08	0.085	0.07
0.75	0.054	0.083	0.088	0.092	0.095	0.095	0.1
1	0.06	0.102	0.106	0.11	0.115	0.115	0.118
1.5	0.08	0.121	0.136	0.15	0.158	0.169	0.182
2	0.105	0.172	0.173	0.19	0.196	0.205	0.215

### **Discussion :**

We used different protein assays such as absorbance at 280 nm , Bradford assay and BCA assay to quantify the protein sample with the help of newly introduced take-3 microplate.

#### **Absorbance at 280 nm :**

Fig 1 shows the graph of concentration vs absorbance. We got absorbance values which were very low to detect.

#### **Bradford assay :**

Fig 2 shows the graph of concentration vs absorbance with incubation time of 5 min. and fig 3 represents represents Bradford assay with 5 and 30 min of incubation times.

In both cases, absorbance values were so lower that it can not be easily detected.

#### **BCA assay :**

Fig 4 is the graph of BCA assay with incubation time of 5 min. This graph shows that for a concentration of 0.0625 mg/ml , the absorbance value was lower. We repeated BCA assay with the same concentration with incubation time of 30 min ( see figure 5 ). It shows that with such low concentration like 0.0625 mg/ml, absorbance was again lower but absorbance values for concentration , ranging from 0.125 to 2 mg/ml , increases to a greater level.

So, BCA assay was repeated with concentration ranging from 0.125 to 2 mg/ml with different experimental conditions. First, BCA assay was conducted at room temperature at intervals of 5 min. In this case, we get  $R^2$  values in the ascending order ( 0.961 , 0.963 , 0.968 , 0.972 , 0.975 ,

0.978 , 0.979 for 0 min , 5 min , 10 min , 15 min , 20 min , 25 min , 30 min respectively ) . Absorbance values also increase from 0 min to 30 min. Fig 6 shows BCA assay at room temperature at intervals of 5 min. Lastly, experiment is performed by maintaining the Take-3 microplate in a humidified incubator at intervals of 5 min. In this case,  $R^2$  values obtained in this case are 0.956 , 0.957 , 0.965 , 0.974 , 0.970 , 0.972 , 0.978 for 0 min , 5 min , 10 min , 15 min , 20 min , 25 min , 30 min respectively. Fig 7 shows BCA assay by heating Take-3 microplate at intervals of 5 min. The data obtained by doing the experiment at room temperature and by doing the same kind of experiment by maintaining the Take-3 microplate in humidified incubator looks similar. Usually BCA assay is performed by heating the microplate in humidified incubator to reduce loss in the sample volume. Increase in temperature increase the absorbance value which decrease the minimum detection level<sup>12</sup>. The data shows that there is not any need to heat the protein sample in incubator prior to experiment or during the experiment. BCA assay with 5 min of incubation time allows the determination of protein concentration.

### **Conclusion :**

We came to conclusion that measurement of protein concentration using Bradford assay and absorbance at 280 nm was not favourable with the help of Take-3 microplate. But BCA assay can be used for the same purpose with protein concentration down to 125 ug/mL, which covers the majority of the samples. The method is safe and uses least amount of time. The method is inexpensive as it involves the use of micropipettes and parafilm”M” and there is not any need for a humidified incubator and disposable microplates. Apart from this, requirements of sample and test reagents were minimum. The results of BCA assay at room temperature at intervals of 5 min and by maintaining Take-3 microplate at 5 min intervals were almost similar, which suggest that there is no need to heat the protein sample for quantitation.

### Future studies :

» Future study will be to lower the volume of the sample from 5 uL to 2 uL to reduce sample use even further.

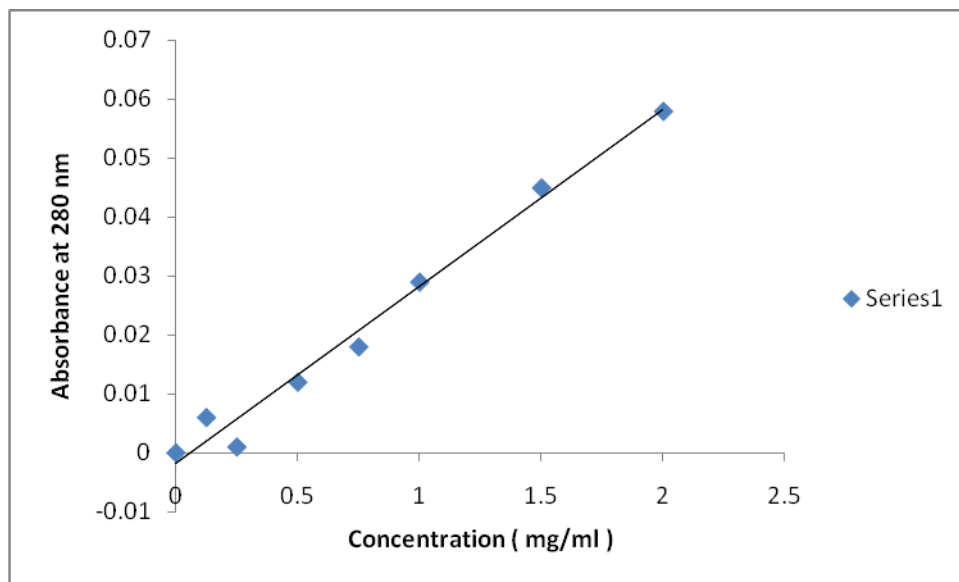
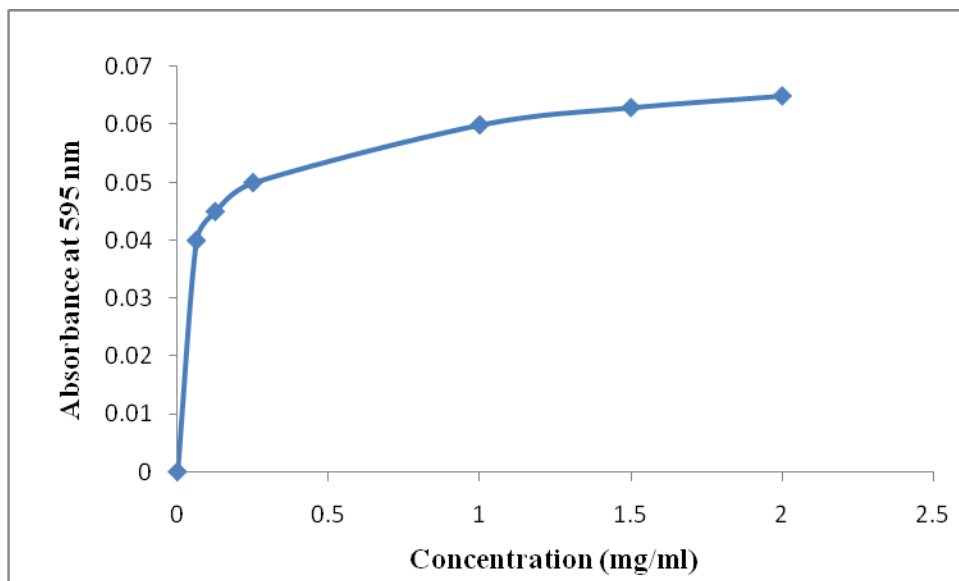
» Test with more users to determine user to user variability.

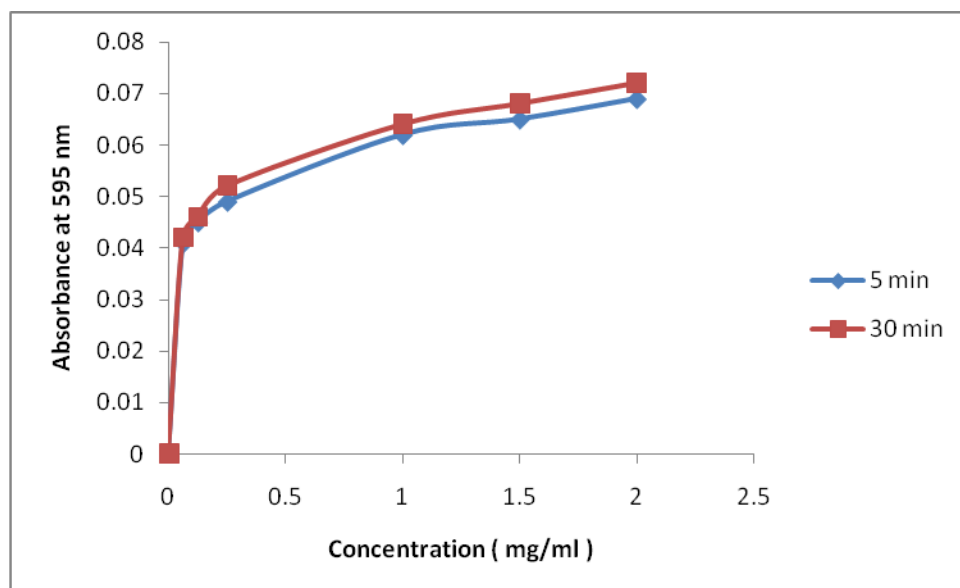
### Funding :

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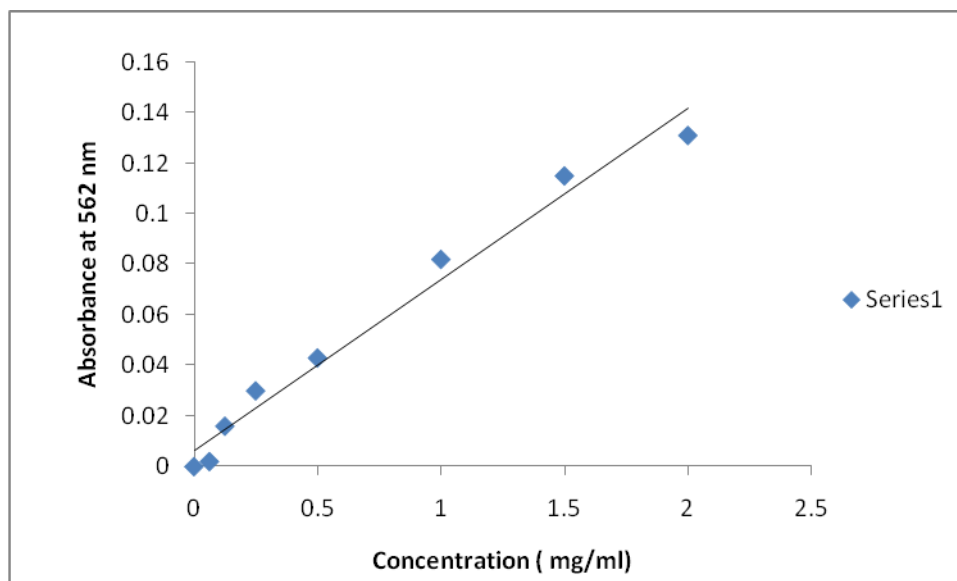
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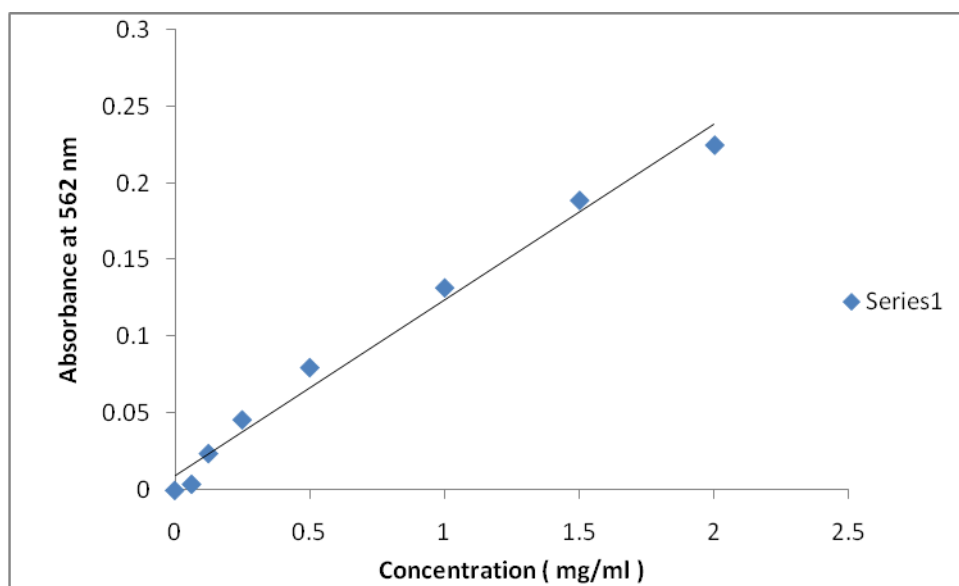
**List of figures :****Fig 1 : Absorbance at 280 nm****Fig 2 : Bradford assay with 5 min of incubation time**



**Fig 3 : Bradford assay with incubation times of 5 min and 30 min**

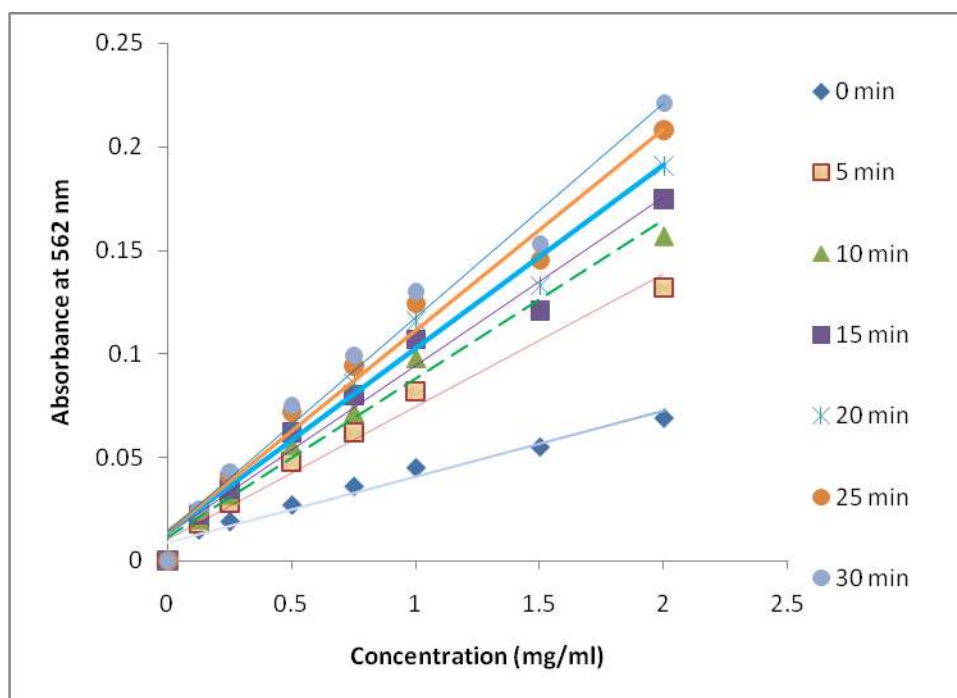


**Fig 4 : BCA assay ( at low concentration ) with 5 min of incubation time**

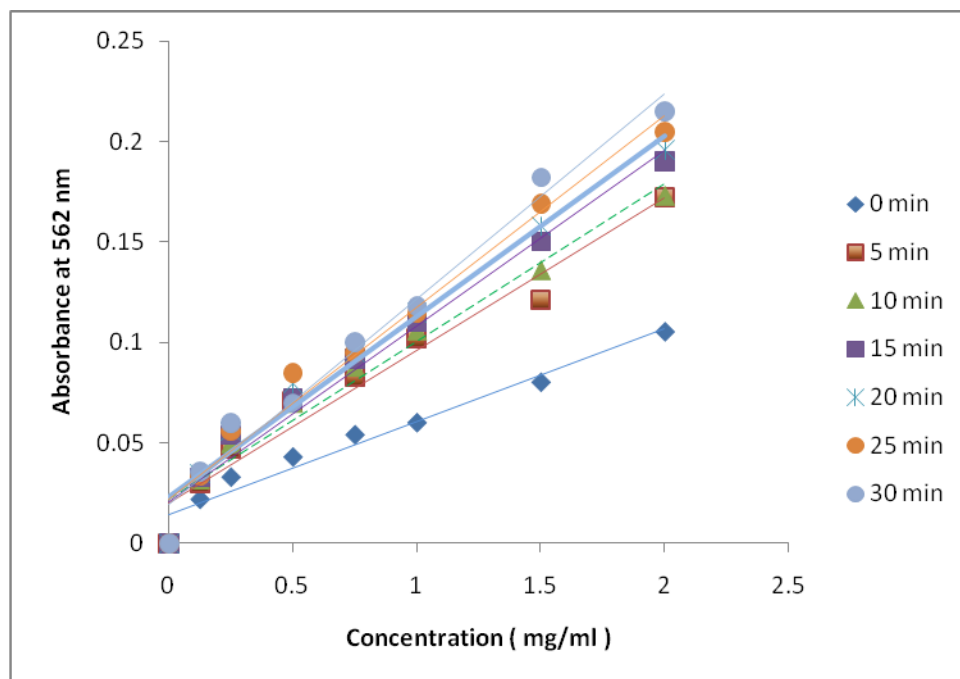


**Fig 5 : BCA assay ( at low concentration ) with incubation time of 30 min**





**Fig 6 : BCA assay at room temperature at the intervals of 5 min**



**Fig 7 : BCA assay in humidified incubator at intervals of 5 min**



**Fig 8 : BSA standards**



**Fig 9 : Epoch microplate spectrophotometer**



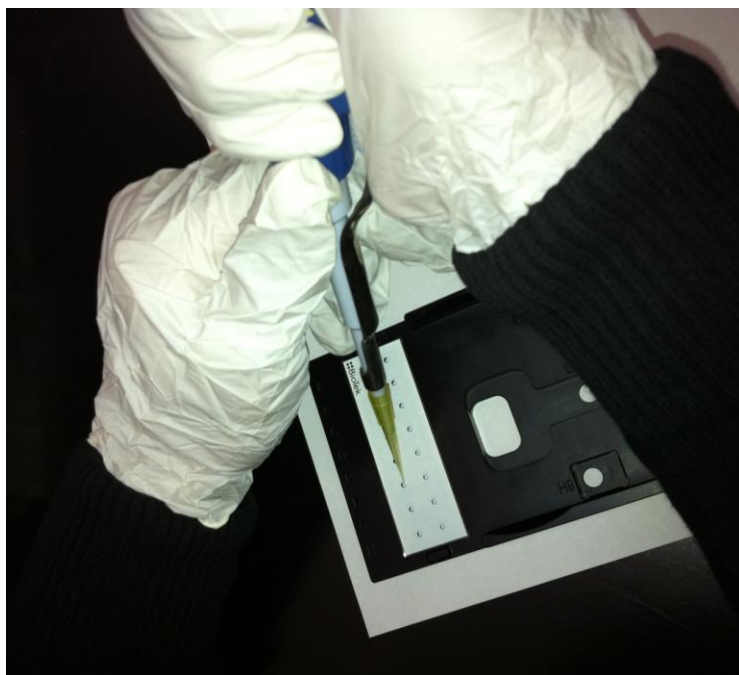
**Fig 10 : Take-3 microplate**



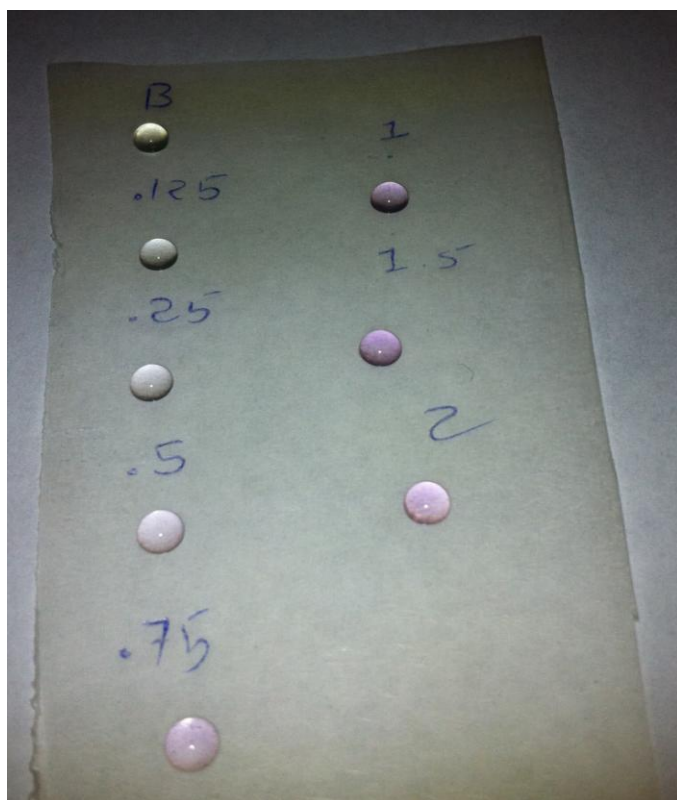
**Fig 11 : Take-3 microplate in humidified incubator**



**Fig 12 : BCA kit and Coomassie reagent**



**Fig 13 : Pipetting microdrops on Take-3 microplate**



**Fig 14 : Protein mixed with BCA reagent on parafilm™M™**

